Detection and Identification of Dizocilpine and its Major Urinary Metabolites in the Horse: A Preliminary Report

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In Honour of Professor Edward Houghton: A Celebration of 30 Years in Racing Chemistry

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Abstract

Dizocilpine ([+]-10,11-dihydro-5-methyl-5H-dibenzo[a,d]cyclohepten-5,10-imine), is a potent and selective NMDA (N-methyl-D-aspartate) receptor antagonist, which acts by blocking receptor ion channels. Dizocilpine is pharmacologically related to ketamine and phencyclidine; as such, it has the potential to affect behavior and performance in horses, with particular efficacy at lower concentrations. We now report development of a sensitive method for the detection of dizocilpine and preliminary characterization of its urinary metabolites in the horse.

Dizocilpine (MW 221) readily produces a protonated species $[M+H]^+$ in formic acid, and yields a m/z 205 product ion in Multiple Reaction Monitoring (MRM), allowing highly sensitive detection of parent drug. The 17 AMU loss most likely represents an unusual loss of CH_5 from the exocyclic methyl group. No unchanged dizocilpine was identified in unhydrolysed urine, and the presence of hydroxymethyl and carboxydizocilpine glucuronide metabolites were supported by observation of m/z 414→238 and 428→235 transitions.

Urine samples from horses dosed with dizocilpine (0.0132 and 0.0656 mg kg^{-1}, iv) were hydrolysed with glucuronidase and were found to contain dizocilpine and OH-dizocilpine. Tentatively identified phase I post-hydrolysis compounds include dizocilpine itself, an hydroxymethyl metabolite, two ring-hydroxylated metabolites, a di-hydroxy metabolite, and a carboxy-dizocilpine metabolite. Corresponding Phase II glucuronidated metabolites were also identified as well as a number of combination metabolites and a posssible n-glucuronide metabolite for a total of at least six identifiable urinary glucuronide metabolites. Among the phase I metabolites, the hydroxymethyl metabolite apparently predominated, especially at the 0.0132 mg kg^{-1} dose.

The goal of this research was to identify a target analyte for dizocilpine in post-administration equine urine, so that work may begin on development of a forensically validated qualitative method for this target analyte. Given the likelihood that the doses of dizocilpine used in attempts to influence the behavior or performance of horses, either alone or in combination with other agents, are expected to be in the order of 0.02 mg kg^{-1} or less, these results suggest selection of the phase I hydroxymethyl metabolite of dizocilpine as the optimal target analyte for regulatory control of dizocilpine in performance horses.

Keywords

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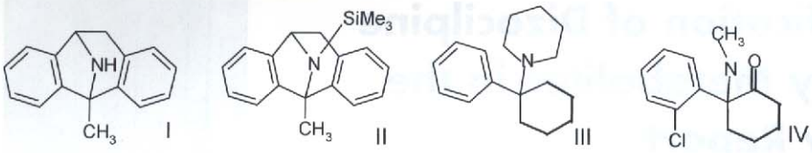

Fig. 1. Dizocilpine (**I**, m.w. 221) and its TMS derivative (**II**, m.w. 293), in comparison with other NMDA-receptor antagonists phencylidine (**III**) and ketamine (**IV**)

Table 1. LC parameters for chromatography of dizocilpine; A (acetonitrile + 0.05% formic acid) and B (deionized water + 5% acetonitrile + 0.05% formic acid)

1 mm column		2 mm column		Solvent composition	
Time, mins	Flow, ml/min	Time, mins	Flow, ml/min	A%	B%
0	0.12	0	0.45	0	100
1.2	0.12	1	0.45	0	100
5	0.12	5	0.45	89.5	10.5
8	0.12	10	0.45	89.5	10.5
8.5	0.12	10.5	0.45	0	100
17	0.12	17	0.45	0	100

Introduction

Dizocilpine (10,11-dihydro-5-methyl-5H-dibenzo[a,d]cyclohepten-5,10-imine (Fig. 1); trade name Merck MK-801) is a potent and selective NMDA (N-methyl-D-aspartate) receptor antagonist thought to act by blocking the receptor ion channel [1]. Dizocilpine shares the property of noncompetitive antagonism of glutamate at NMDA receptors with phencyclidine and ketamine. As a result, these agents share the properties of dissociative anesthesia and the ability to serve as reinforcing stimuli [2]. Following IV administration in rhesus monkeys, the reinforcing property occurs immediately with ketamine, within 3–10 min with phencyclidine, and after 30 min with dizocilpine [2]. These compounds are structurally similar (Fig. 1), and molecular modeling studies indicate a unifying pharmacophore in the specific orientation of the nitrogen lone electron pair relative to the phenyl group [3].

Abuse of NMDA receptor antagonists by humans can lead to psychotomimetic and cognitive deficits that resemble the symptoms of schizophrenia [4], which led to suggestions that reductions in NMDA receptor function contribute to the pathology of schizophrenia [5]. Degeneration of striatal neurons induced by NMDA antagonists or quinolinate can be prevented by systemic administration of dizocilpine up to five hours after excitotoxin injection. There is an indication that dizocilpine has rather slow kinetics during channel blockade, possibly owing to high rates of bound versus free concentrations (60% bound in serum and 90% in brain). Effective treatment may therefore depend on therapeutic drug monitoring, and sensitive gas chromatographic methods have been developed for its quantitation in animal models to a lower limit of quantitation (LOQ) of approximately 0.1 ng mL^{-1} [6, 7]

NMDA antagonist actions have been studied in rats and mice and include increase in motor behaviors such as overall activity, head twitch and head weaving, which can be likened to motor abnormalities associated with phencyclidine administration in humans [5] Brief NMDA receptor blockade with dizocilpine during rat neonatal development can yield chronic behavioral changes, including increased locomotor activity in adults [8]. Systemic administration of (+) MK-801 (dizocilpine maleate) causes both neuroprotective and neurotoxic activities depending on doses and conditions [9].

In the horse, dizocilpine is apparently a potent pharmacological agent, with doses of 0.0656 mg kg^{-1} rapidly inducing diminution of locomotor activity and behavioral sensitization. Additionally, it is possible that dizocilpine, in common with other end NMDA antagonists, may affect equine behavior at relatively low doses. Because of dizocilpine's potential for modifying locomotor activity and alleviating spinal transmission of pain, and despite its current lack of classification by the Association of Racing Commissioners International (ARCI Uniform Classification Guidelines, 2003), it likely bears the prospect of abuse potential in performance horses, particularly those whose behavioral repertoire or clinical circumstances may require an alleviation of pain perception or subtle behavioral changes. In that regard, injection of dizocilpine into the knee joints of rats prior to carrageenan injection significantly prevented pain related behavior [10]. The overall goal of this research is to identify a suitable target analyte for dizocilpine in horse urine to enable development of a forensically validated qualitative analytical method for this target analyte in performance horse urine suitable for use in its regulatory control .

Experimental

Horses

Eight mature thoroughbred mares weighing 476–532 kg were fed twice a day with grass hay and feed (12%), which was a 50:50 mixture of oats and an alfalfa-based protein pellet. The animals were vaccinated annually for tetanus and were dewormed quarterly with ivermectin (MSD Agvet, Rahway, NJ, USA). A routine clinical examination was performed before each experiment to assure that the animals were healthy and sound. During experimentation, horses were provided water and hay *ad libitum*. Each mare served as its own control. Animals used in these experiments were managed according to the rules and regulations of the Institutional Animal Care Use Committee at the University of Kentucky, which also approved the experimental protocol. For analytical purposes, dizocilpine (0.0132 or 0.0656 mg kg^{-1}, iv) was administered to 2 horses I.V. by dissolution of the Sigma standard in DMSO (0.5 mL) and saline (2.5 mL). Urine samples were collected immediately before and at 1, 2, 4, 6, 8, 24 and 48 h after administration using a Harris flush tube (24 Fr × 60 in; Seamless, Ocala, FL, USA). Urine samples were divided into aliquots stored at −20 °C until assayed. To measure pharmacological effects, dizocilpine (0.0264 mg kg^{-1} in 0.5 mL DMSO and 2.5 mL saline) was administered to six horses. Our source

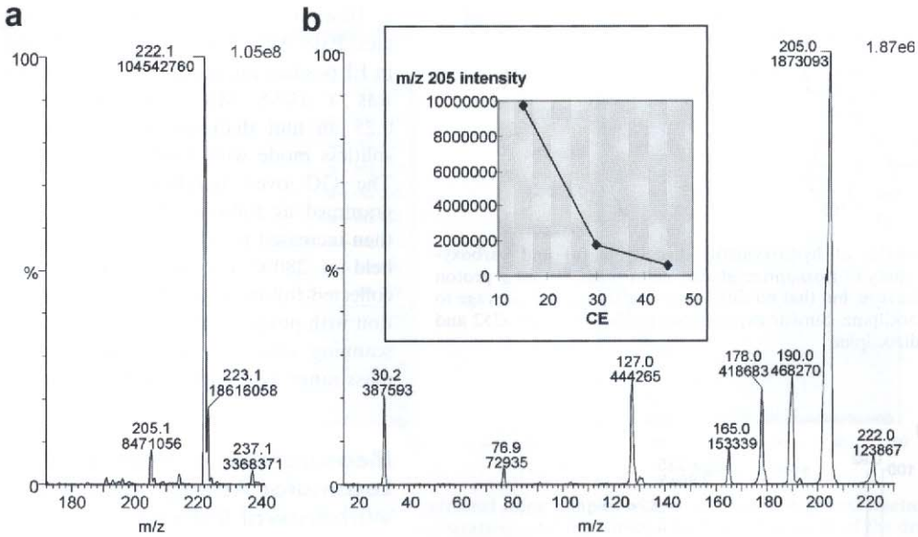

Fig. 2. Dizocilpine ESI(+) mass spectrometry. (a) shows 1 ug mL^{-1} dizocilpine in 0.05% formic acid: acetonitrile, 1:1 examined by direct infusion-ESI(+)MS. (b) shows a product ion spectrum derived from the *m/z* 222 ion seen in the left panel and examined at collision energy set at 30 V. Peak assignments: *m/z* 205, loss of CH_5; 190 = 205 minus [CH_2 +H]; 178 = 205 minus [CH=CH +H]; 165 = 205 minus [CH=CH-CH +H]; rest unassigned. The inset figure displays the variation of *m/z* 205 product ion intensity with collision energy for CID

of dizocilpine was Sigma (Lot# 083K4610) with a certificate of analysis indicating an estimated purity of 100%, which identity and purity was confirmed by in-house GC-MS evaluation.

Sample Preparation and Analysis

For beta-glucuronidase hydrolysis, urine samples were treated for 3 h at 65 °C with *Patella vulgata* beta-glucuronidase (1000 units of Sigma Type L-II mL^{-1} urine adjusted to pH 5 with 0.25 M sodium acetate) [11]. The resultant hydrolysates were centrifuged 5 min at approx 700 × g to remove sediment, and the decanted liquid was subjected to solid-phase extraction (SPE).

SPE was performed on a Speedisk 48 Pressure Processor (SPEware Corp., San Pedro, CA, USA). SPE columns type CSDAU Clean-screen (United Chemical Technologies, Bristol, PA, USA) were conditioned by adding sequentially 3 mL methanol, 3 mL water, and 1 mL 0.1 M sodium phosphate buffer (pH 6.0). Samples brought to pH 6.0 with 1 mL 0.1 M sodium phosphate buffer were loaded, then the column was washed sequentially with 2 mL water, 2 mL 1M acetic acid, 4 mL methanol. Then the column was eluted with 3 mL dichloromethane/isopropanol/NH_4OH (concentrated) (78:20:2, *v:v:v*) into glass tubes. The eluent was evaporated to dryness under a stream of N_2 in a 40 °C water bath. The residue was dissolved directly in 1 mL acetonitrile: 0.05% formic acid, 1:1, then transferred to a micro-injection vial and sealed. Then 10–20 µL were injected into the Hewlett-Packard 1050 HPLC– Micromass Quattro II ESI(+)MS-MS.

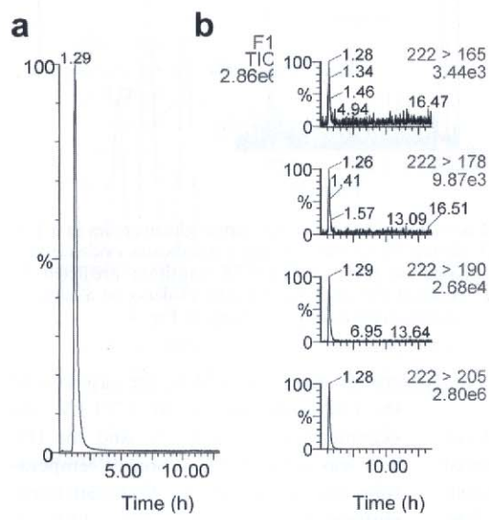

Fig. 3. Dizocilpine LC-MS-MS. A 0.3 µL sample of 10 ng μL^{-1}3 (3 ng $column^{-1}$) was injected and subjected to gradient chromatography as described by Table 1 for the 1 mm column. (a) represents the total ion chromatogram, whereas (b) depicts coelution of qualifier (*m/z* 165, 178, 190) and quantifier (*m/z* 205) ion data arising at the expected retention time

Extracts were also infused at 1.2 mL h^{-1} via a Harvard syringe pump equipped with a 500 µL Hamilton gas-tight syringe directly into the electrospray probe of the Quattro II MS-MS (Micromass, Beverly, MA, USA). Full scan electrospray ionization (ESI+) mass spectra were obtained on analytical dizocilpine standards at 1 µg mL^{-1} in 1:1 acetonitrile: 0.05% formic acid (aq), pH~4, and on urinary extracts, both by direct infusion. All spectra were optimized by combination of 1–2 min of uniformly acquired data, background subtraction, and peak smoothing.

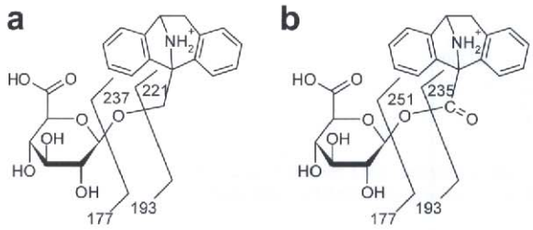

Fig. 4. Predicted structures for glucuronides of hydroxymethyl-dizocilpine (a) and carboxy-dizocilpine (b). Analogy to results from study of isoxsuprine glucuronides suggests that a proton shifts to give m/z 414→238 for the first cleavage, but that no shift occurs for the second cleavage to give m/z 414→221 for hydroxymethy-dizocilpine. Similar expectations predict m/z 428→252 and 428→235, respectively, for the carboxy-dizocilpine

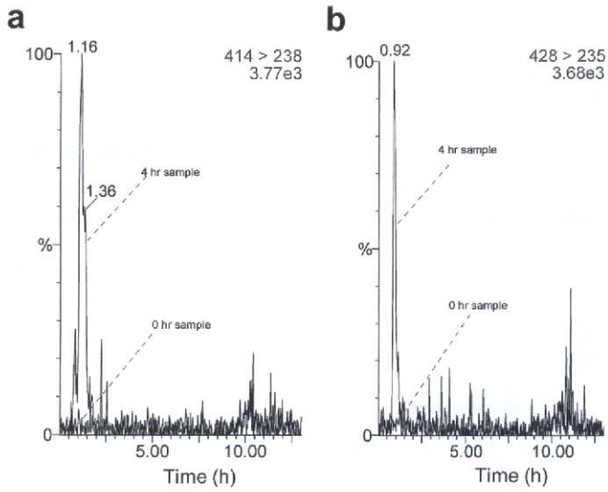

Fig. 5. HPLC-ESI(+)-MS-MS for MRM detection of potential dizocilpine glucuronides in a 4 hr urine sample following 0.0656 mg kg-1 IV dosing on a 1 mm column. (a) indicates evidence of a hydroxymethyl (or isomer) glucuronide, based on the m/z 414→238 transition predicted by reasoning of Fig. 4; 0 and 4 h results are overlayed. (b) similarly indicates evidence of a carboxy glucuronide, based on the m/z 428→235 transition predicted by reasoning of Fig. 4

MS-MS Tuning and HPLC

The mass spectrometer was tuned for positive ion spectra by direct infusion of 1 μg mL^{-1} dizocilpine in acetonitrile:0.05% formic acid (aq), 1:1. The peak shape and intensity of the mono-protonated dizocilpine m/z 222 ion were optimized by adjustment of capillary, HV lens, cone voltage, skimmer lens, and RF lens settings. Skimmer lens offset was left at 5 V. Collision gas (argon) and collision energy were adjusted for collisionally-induced dissociation (CID) in the central hexapole by optimizing settings as needed for the second quadrupole. Generally, the collision gas was set to $1–3 \times 10^{-3}$ mbar. Increasing the photomultiplier setting 100–150 V above the regular 650 V sufficiently increased sensitivity. In general for positive mode, the source cone voltage was set 20–24 V, the collision energy was set at −24 V, the capillary of the ESI probe was set at +3.1 kV, the skimmer was set at 1.6 V, and the HV lens was set at 0.5 kV. Source temperature was set at 120 °C. Gradient chromatography with the HPLC-ESI(+)-MS-MS utilized the settings for solvents A (acetonitrile + 0.05% formic acid) and B (deionized water + 5% acetonitrile + 0.05% formic acid) shown in Table 1.

GC-MS Analysis

Dizocilpine standard and beta-glucuronidase hydrolyzed urine extracts were also derivatized for GC-MS analysis by resuspension in 50 ul BSTFA + 1% TMCS (Pierce Chemical Co., Rockford, IL, USA) plus 15 uL N,N-dimethylformamide with reaction at 75 °C for 30–40 min [11]. Analyses were performed on a Hewlett-Packard (Agilent Technologies, Palo Alto, CA, USA) 5890 GC-MS in EI positive ion mode. The GC column was a HP-5 MS, 30 m × 0.25 mm × 0.25 μm film thickness operated in the splitless mode with 1 mL min^{-1} helium. The GC oven temperature was programmed as follows: 180 °C for 2 min, then increased to 280 °C at 20 °C min^{-1}, held at 280°C for 10 min. Data were collected following a splitless 1 μL injection with purge on at 2 min. For GC-MS scanning experiments, the m/z 50–700 mass range was scanned at 1.19 scan s^{-1}.

Measurement of Heart Rate, Locomotor Activity, and Hoof Withdrawal Reflex Latency

Heart rate (HR) and locomotor activity was simultaneously monitored in each of six horses. Heart rates were recorded at 1 min intervals during each experiment by an on-board heart rate computer (Polar CIC Inc, Port Washington, NY, USA). An elastic strap with a receiver and transmitter attached was placed around the chest of the horse. The transmitter was connected to two electrodes placed on shaved areas of the sternum and left side of the anterior chest. Electrode gel was used to insure proper conduction of the HR signal.

The locomotor chambers are two 3.4 × 3.4 m box stalls equipped with Minibeam sensors (SM31E and SM2A31R, Banner Engineering, Minneapolis, MN, USA) spaced equally around the stall 45 cm above the floor. Each time the horse disrupted the beam of light, an interruption was scored, and this output was summed and recorded on a data logger (CR10, Campbell Scientific, Inc., Logan, UT, USA).

Behavioral experiments followed a rigorous standard protocol to reduce variability from extraneous effects. A horse was placed in each behavior stall at 0700 h, and the HR strap was attached. The horse was allowed to acclimate to the stall for 7 h. Recording of locomotor and HR activities was begun at 1500 h. Baseline activity was recorded for 10 min, then the control and experimental treatments were administered. Locomotor and HR data were recorded for 14 h until 0500 h the following morning. The total number of interruptions was summed every 15 min. Both control and experimental trials were run simultaneously.

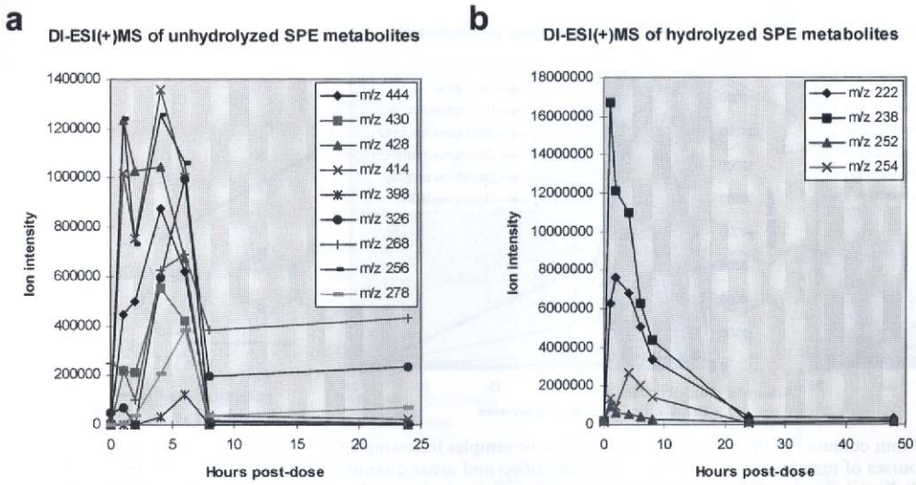

Fig. 6. Direct-infusion ESI(+)MS of solid-phase extracted urine samples without (a) and with (b) enzymatic hydrolysis, indicating the relative time course of metabolism, with urinary metabolite concentrations peaking within 6 h of administration of the drug

To assess any local anesthetic effect, focused radiant light (heat) was used as a noxious stimulus and was directed onto the fetlock of a horse to elicit the classic flexion-withdrawal reflex [12]. Hoof withdrawal reflex latency (HWRL) is defined as the time between lamp illumination and withdrawal of the hoof. The intensity of the light beam was adjusted so that HWRL period was about 3–4 sec, with the actual HWRL recorded on an electronic timer built into the lamp. In the anesthetized leg, the duration of light exposure was limited to 10 s to prevent undue damage to the skin. A secondary unfocused light beam (sham light) was used to confound the horse, reducing the possibility that the flexion-withdrawal reflex was to visual rather than thermal perception of the focused light beam.

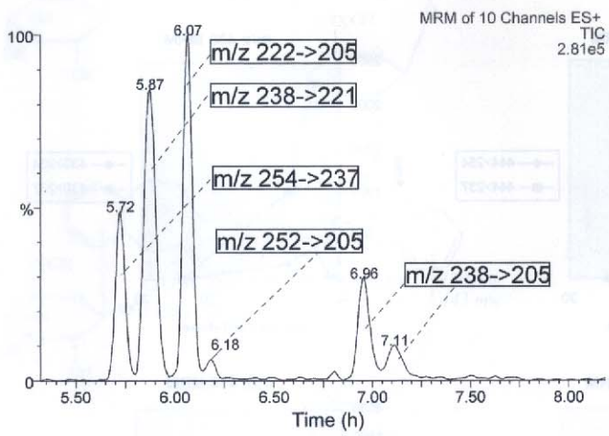

Fig. 7. LC-ESI(+)MS-MS of dizocilpine (0.0656 mg kg^{-1}) hydrolyzed urine SPE extract 4 hours post-dose, applying MRM transitions as indicated. The 2 mL urine extract was resuspended in 1 mL acetonitrile: 0.05% formic acid prior to HPLC

Results

Ionization

Dizocilpine readily produced a $[M+H]^+$ species in 0.05% formic acid at *m/z* 222, with a small amount of in-source fragmentation to *m/z* 205 (Fig. 2 [left]). The product ion spectrum contained a base peak at *m/z* 205, representing an unusual loss of 17 amu. Loss of ammonia (NH_3 = 17 m·w.) as a neutral fragment seemed possible, although *in silico* fragmentation by ACD/MS Fragmenter (Advanced Chemistry Development, Toronto, Ontario, Canada) software favored loss of an unusual CH_5 (or methane plus a proton) group from the pseudomolecular $[M+H]^+$ ion. The latter interpretation is supported by subsequent indications of metabolism specifically at the exocyclic methyl group, which transformations selectively removed capacity for pronounced loss of 17 amu. However, further studies are necessary to fully characterize this 17 amu loss.

Detection by LC-ESI(+)MS-MS

Low collision energy settings maximized the intensity of the *m/z* 205 peak for highly sensitive detection of dizocilpine by MRM. Such detection afforded reproducible chromatography with early elution on a Luna 30 × 1 mm × 3 µm phenyl-hexyl column (Phenomenex, Torrance, CA, USA) (Fig. 3). Optimised detection involved MRM with *m/z* 222 yielding ions of 205, 190, 178, and 165 with a dwell time of 0.02 s, 28V cone voltage, and a collision energy of 13 V. The linearity of dizocilpine response was assessed and found to become curvilinear at quantities of 20 ng on-column and higher. This method provided excellent response, even at the lowest injection of 0.1 ng $column^{-1}$, indicating the possibility of a very sensitive detection method for parent dizocilpine.

Metabolites/Unhydrolysed Urine

Analogy to human metabolism of the exocyclic methyl group of delta-9-tetra-

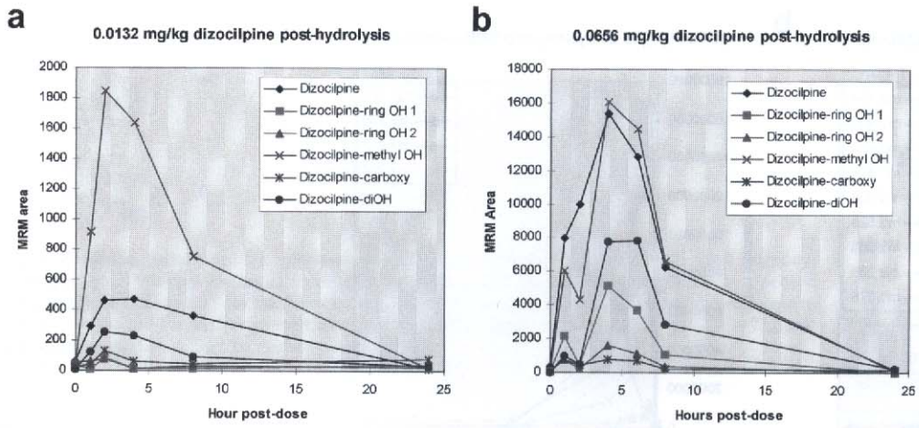

Fig. 8. HPLC-ESI(+)MS-MS with the 2 mm column of SPE-extracted hydrolyzed urine samples following 0.0132 mg kg^{-1} (a) and 0.0656 mg kg^{-1} (b) I.V. doses, indicating the relative time courses of metabolism. Compounds were identified and areas quantitated by focusing on the following MRM transitions (retention time in parentheses): dizocilpine: *m/z* 222- > 205 (6.1 min); dizocilpine-ring hydroxyl #1, *m/z* 238- > 205 (7.0 min); dizocilpine-ring hydroxyl #2, *m/z* 238- > 205 (7.1 min); dizocilpine-methyl-OH, *m/z* 238- > 221 (5.9 min); dizocilpine-carboxy, *m/z* 252- > 205 (6.2 min); dizocilpine-diOH, *m/z* 254- > 237 (5.7 min)

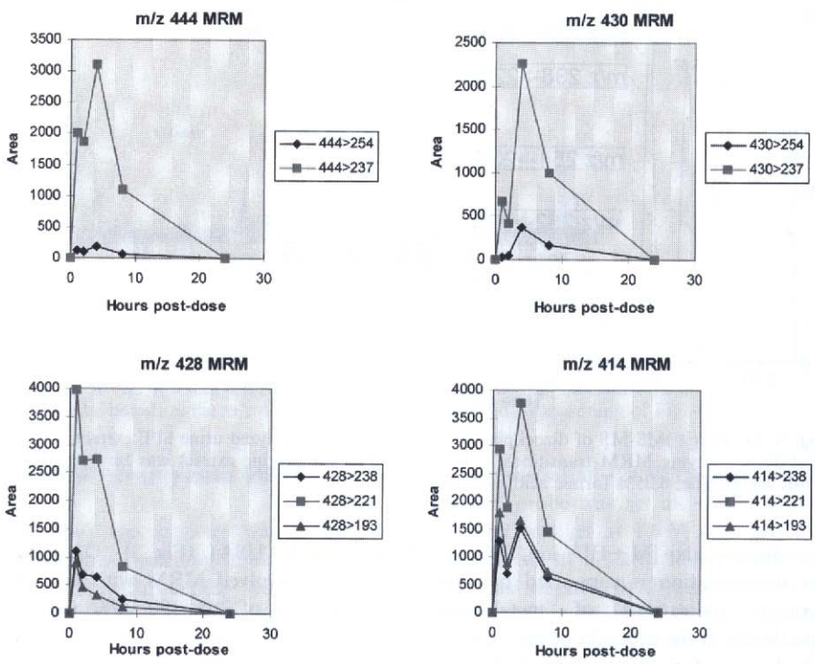

Fig. 9. HPLC areas for dizocilpine-derived glucuronides as measured by ESI(+)MRM of transitions as indicated. Plots show trends over the time course post-50 mg dose, and generally demonstrate correspondence of behavior for given transitions from a given parent ion

hydrocannibinol to hydroxymethyl and carboxy functionalities [13] suggested similar routes of metabolism for dizocilpine. In addition, analogy to recently determined fragmentation patterns of isoxsuprine metabolites, including isoxsuprine glucuronide [14] and methylated isoxsuprine glucuronide, suggested possible cleavages on either side of the glucuronide-O-linkage of potential dizocilpine glucuronides (Fig. 4).

Analysis of unhydrolyzed equine urine samples following a 50 mg IV administration of dizocilpine was carried out by centrifugal filtration of the urine, followed by dilution 1:10 into acetonitrile:0.05% formic acid (aq) for direct infusion-ESI(+)-MS analysis. These direct infusion studies were of insufficient sensitivity to detect any major differences between the 0 h sample and time points 1, 2, 4, 6, 8, 24 or 48 h post-dose. However, based on predicted cleavage events shown in Fig. 4, useful results were obtained by MRM analysis with HPLC sample introduction on a 1 mm column. These approaches showed, first of all, that parent dizocilpine was not detected at any concentration in any of the unhydrolyzed samples (data not shown). Additionally, initial chromatographic evidence for glucuronides was found by MRM, as shown in Fig. 5, supporting the structures suggested in Fig. 4. Fig. 6 shows that unhydrolyzed and hydrolyzed urine SPE extracts examined by direct infusion-ESI(+)-MS contained a number of peaks that varied over the time course of sample collection, and as such represent candidate urinary metabolites of dizocilpine. Preliminary centroid data-type product ion spectra on direct infused SPE extracts suggested a number of MRM transitions that could appropriately be monitored for maximum sensitivity HPLC assays with ESI(+)MS-MS detection (data not shown).

Metabolites/hydrolysed Urine

Application of a number of MRM transitions to hydrolyzed urine extracts is depicted in Fig. 7. These data suggest at least six putative candidate post-hydrolysis urinary metabolites, of unprotonated molecular weights 221, 237 [3 types], 251 and 253. The raw MRM areas for these compounds and their transitions are shown in Fig. 8 for the 0.0132 and 0.0656 mg kg^{-1} doses of dizocilpine. As

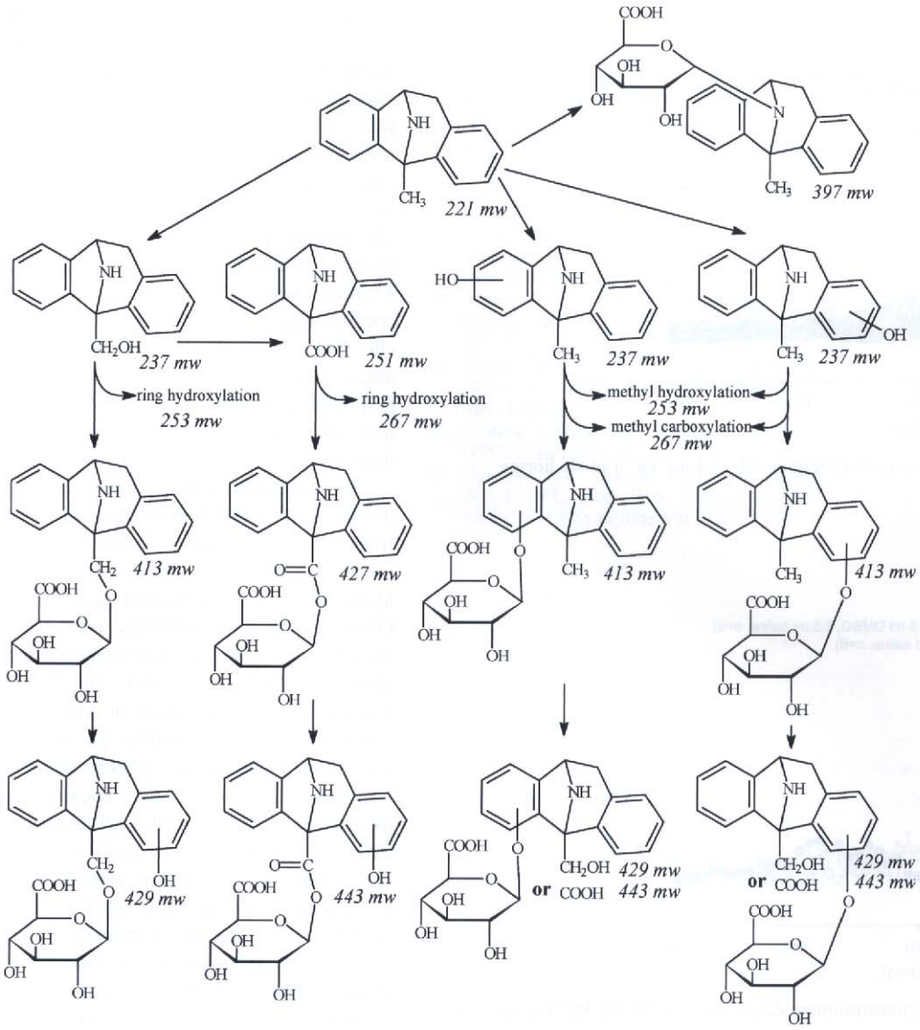

Fig. 10. Summary of proposed dizocilpine metabolism. Pathways are intended to summarize probable product structures. Molecular weight species observed as $[M+H]^+$ ions during this research include: 237, 251, 253, 255 (structure not finalized and not included above), 267, 413, 427, 429, and 443 MW. There is also more tentative support for the MW 397 species as well

would be expected, there is a delay in the time to metabolite peaks following the 0.0656 mg kg^{-1} dose as compared with the 0.0132 mg kg^{-1} dose, as well as a shift in the apparent relative prominence of individual metabolites.

Fig. 9 shows the raw data for excretion of glucuronide metabolites. The legends reflect the loss of fragments from the respective molecular ions $[M+H]^+$. The concentration of the metabolites peaks at about 5 h post-dosing (0.0656 mg kg^{-1}) as do the non-glucuronide metabolites in Fig. 8. Fig. 10 presents a summary overview of the probable urinary metabolites of dizocilpine in the horse. All metabolite identifications are tentative at this point, but the apparent predominance of the hydroxymethyl dizocilpine metabolite strongly suggests this metabolite as the optimal target analyte for regulatory control of dizocilpine in the horse.

Preliminary Evaluation of Pharmacodynamic Activity in the Horse

Administration of dizocilpine at a dose of 0.0656 mg kg^{-1} rapidly reduced the observed locomotor activity, with the horse becoming immobile, difficult to approach, and apparently hypersensitive to touch. This diminution of locomotor activity and sensitization commenced about 5 min after dizocilpine administration, peaked rapidly and had essentially disappeared by 30 min after administration. Evaluation of the overall clinical response to this dose suggested that 0.0656 mg kg^{-1} is a substantial dose, whereas few overt behavioral effects were observed after the 0.0132 mg kg^{-1} dose. Preliminary evaluation of hoof withdrawal reflex time to a nociceptive stimulus revealed no significant effect of this dose (0.0132 mg kg^{-1}) of dizocilpine.

At a lower dose of 0.0264 mg kg^{-1}, there was a significant increase in heart rate following dizocilpine administration. Fig. 11 shows that heart rate increased rapidly, and the significant increase persisted for about an hour following intravenous administration. Although the trend suggests a slight increase with this low dizocilpine dose, behavior activity was actually not significantly different between the control and treatment groups (Fig. 12). Overall, the observed pharmacodymamic response to the

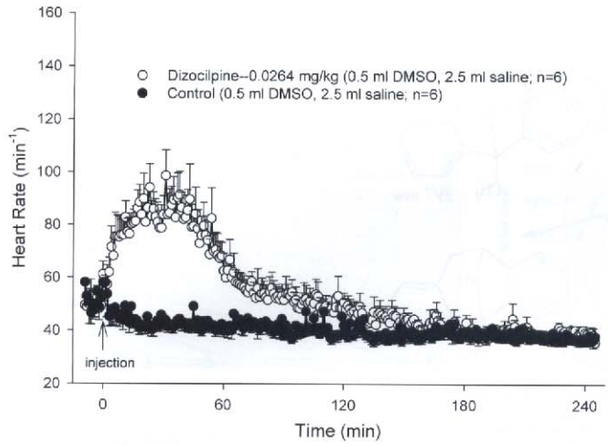

Fig. 11. Heart rate following administration of dizocilpine (0.0264 mg kg^{-1}) to six horses

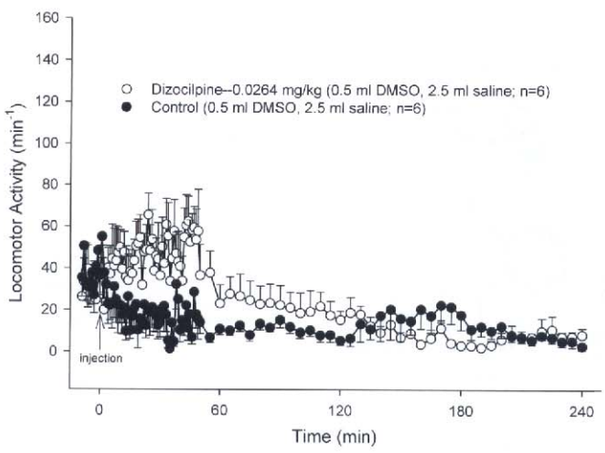

Fig. 12. Locomotor activity following administration of dizocilpine (0.0264 mg kg^{-1}) to six horses. Error bars represent standard error

0.0656 mg kg^{-1} dose was generally consistent with what may be expected from a ketamine-like agent.

Discussion

Review of the structure (Fig. 1) and pharmacology of dizocilpine shows that it is a potent and selective NMDA (N-methyl-D-aspartate) receptor antagonist, pharmacologically related to ketamine and phencyclidine; as such, it is likely to affect pain perception or behavior in performance horses. Beyond this, there is reason to assume that its effects on equine behavior may occur at relatively low concentrations of dizocilpine. We therefore undertook the development of a highly sensitive method for the detection of parent dizocilpine and preliminary characterization of its urinary metabolites in the horse. The goal of this research was to identify a suitable target analyte for dizocilpine in horse urine so that a forensically validated qualitative method suitable for regulatory control of this agent might be developed. The search for and determination of this target analyte was conducted using urine from horses having received doses of the drug producing low or minimal pharmacological activity (Fig. 12).

The first step was to identify chromatographic conditions suitable for identification of a sensitive and specific detection method for dizocilpine itself. As set forth above, dizocilpine readily produced a + 1 protonated species in 0.05% formic acid at m/z 222. Furthermore, at low collision energies, it yielded a product ion spectrum with a base peak at m/z 205. This mass change representing a loss of 17 amu indicates the possibility of a highly sensitive method of detection for dizocilpine by MRM. As noted previously, this 17 amu loss also suggests an unusual CH_5^+ (or methane plus a proton) group loss from the pseudomolecular $[M+H]^+$ ion, which interpretations are supported by other indications of metabolism at the exocyclic methyl group, and loss of which group selectively reduces observed 17 amu losses. It should be noted that cocaine and its metabolites have a bicyclic NH similar to that of dizocilpine and exhibit no significant loss of 17 amu [15], although this still does not rule out loss of a neutral NH_3 molecule for dizocilpine.

At low doses of dizocilpine, the predominant Phase I metabolite recovered from beta glucuronidase hydrolyzed urine is the MW 237 species, tentatively identified as hydroxymethyl dizocilpine. Other Phase I metabolites tentatively identified include a methyl carboxylated species (MW 251), and two ring hydroxylated species, each of MW 237. These Phase I metabolites provide at least four identifiable glucuronidation opportunities and yield respectively dizocilpine hydroxymethyl glucuronide (MW 413), dizocilpine carboxy-glucuronide (MW 427), and two distinct ring hydroxylated glucuronide species, each of MW 413. Various combinations of these Phase II metabolites, with other Phase I functional groups yield species of MW 429, 443 and other molecular weight isomers of these metabolic species. As noted in the results section, evidence also exists for the presence of an unusual N-glucuronide of dizocilpine, MW 397.

In summary, after IV administration, dizocilpine (0.0132 and 0.0656 mg kg^{-1}) is metabolized to at least four identifiable Phase I products, involving, respectively, methyl hydroxylation, methyl carboxylation and two ring-hydroxylation products. These four Phase I metabolites are then glucuronidated to yield their respective Phase II glucuronide metabolites. A number of other species, which represent various combinations of the above metabolic species, are also identifiable. In this regard, the metabolism of dizocilpine is similar to that of cocaine in that several cocaine metabolites show ring hydroxylation at multiple sites [15]. Additionally, there is preliminary evidence suggesting the direct glucuronidation of dizocilpine at the amino group, a

metabolic process for which precedent exists in the literature [16–19].

The identification of the hydroxymethylated product of dizocilpine as the predominant equine urinary metabolite after administration of a 0.0132 mg kg^{-1} dose of this agent suggests that this metabolite is the optimal target analyte for regulatory control of dizocilpine in performance horses.

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