JOURNAL OF APPLIED TOXICOLOGY
J. Appl. Toxicol. **22**, 117–121 (2002)
Published online in Wiley InterScience (www.interscience.wiley.com). DOI:10.1002/jat.836

Determination of the Highest No-effect Dose (HNED) and of the Elimination Pattern for Cocaine in Horses

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Key words: cocaine; horses; highest no-effect dose; locomotor activity; pharmacokinetics.

Cocaine is one of the most widespread illegal stimulants utilized by the human population throughout the world. The aim of this study was to establish the highest no-effect dose (HNED) of cocaine on the spontaneous locomotor activity (SLA) of horses in a behavior chamber, and thereby to determine the maximal acceptable threshold of the urinary drug concentration in horses. Twelve English thoroughbred mares received 0.02, 0.03, 0.04, 0.08 or 0.12 mg kg^{-1} cocaine i.v. or saline solution (control). It was noted that doses above 0.04 mg kg^{-1} induced a significant increase in SLA ($P < 0.05$, Tukey's test). No significant increase in SLA was seen in the mares that received 0.03 mg kg^{-1}, but the animals showed important behavioral changes that did not occur after the 0.02 mg kg^{-1} dose. It was concluded that the HNED of cocaine for horses in a behavior chamber is 0.02 mg kg^{-1}. After injection of this dose in five horses, urine samples were collected at predetermined intervals through vesical catheterization. The concentrations of cocaine, norcocaine, benzoylecgonine and ecgonine methyl ester were quantified by liquid chromatography/electrospray ionization tandem mass spectrometry. Cocaine and norcocaine concentrations remained consistently below the level of detection. Benzoylecgonine reached a mean (± SEM) maximum concentration of 531.9 ± 168.7 ng ml^{-1} after 4 h, whereas ecgonine methyl ester peaked 2 h after injection at a concentration of 97.2 ± 26.5 ng ml^{-1}. The maximum admissible concentration for cocaine and/or metabolites in the urine of horses is difficult to establish unequivocally because of the substantial individual variation in the drug elimination pattern observed in horses, which can be inferred by the large standard error of the means obtained. Copyright © **2002 John Wiley & Sons, Ltd.**

INTRODUCTION

Cocaine is an alkaloid derived from the leaves of the coca plant *Erythroxylum coca*, which grows in the Andes Mountains in western South America. It is currently the major drug of abuse in humans and is widely available among certain groups. It has a long history of use as a stimulant drug and has been used for millennia in South America to prolong endurance and improve work output.

Most the effects of cocaine on the central and autonomic nervous systems are mediated through alterations in neurotransmission. The neurotransmitters dopamine, norepinephrine and serotonin, which are closely associated with the expression of behavior and emotions, are the ones predominantly affected by cocaine use.[1]

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Contract/grant sponsor: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).
Contract/grant sponsor: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Systemic effects of cocaine predominantly occur from the intense activation of the sympathetic nervous system, due to the blockade of neurotransmitter reuptake, which constitutes the principal means by which the neurotransmitter effects are terminated. This blockade results in a rise in neurotransmitter concentration in the synaptic cleft, thus producing a sustained action on the receptor system. These include tachycardia, hypertension, mydriasis, vasoconstriction, diaphoresis and tremor. In addition, the norepinephrine system in the hypothalamus regulates appetite, thirst, body temperature, sleep and sexual arousal.[2] Euphoria, an effect largely due to enhanced stimulation of the dopaminergic system, is the most prominent central nervous system effect of cocaine intake. All these pharmacological actions of cocaine are well described and its potential for abuse in racehorses is clear-cut.

Studies of the effects of cocaine on treadmill exercise in horses[3] showed that cocaine administration increased the maximal heart rate but had no effect on atrial or ventricular function during exercise. A 200-mg dose of cocaine increased the mean arterial blood pressure at rest and during exercise and also influenced the central nervous system, masking fatigue and allowing the horses to tolerate greater work intensities and blood lactate concentrations.

Received 31 August 2001
Revised 29 October 2001
Accepted 29 October 2001

The effects cited above are strongly suggestive of positive performance effects.

Drug testing procedures now are more sophisticated than they used to be. With the introduction of high-sensitivity immunoassays into race testing, it has become clear that low concentrations of cocaine or its metabolites are found not uncommonly in post-race urine samples. In latter years, some jurisdictions have reported an excessive number of cocaine-positive tests in samples of horse material. Nowadays, however, it is exceptionally difficult to determine the pharmacological or forensic significance of residues of this drug in equine samples. More recently, reports from regulatory professionals suggest that cocaine may be applied to tongue ties in an attempt to influence the performance of the horses. These applications apparently produce small or undetectable concentrations of cocaine in urine.

On the other hand, it was demonstrated that the presence of relatively large concentrations of benzoylecgonine could be detected in the urine of humans who had ingested coca tea containing not more than 2.15 mg of cocaine.[4] Cocaine- positive urine samples were found also after exposure to small amounts of the drug via the oral route.[5] These findings are indicative of the problems that toxicologists and forensic scientists could face when interpreting data of cocaine metabolite contents in urine.

Cocaine is thought to be well absorbed after administration in humans by all other routes, including coca chewing and nasal applications,[6] smoking,[7] dermal application[8] and after lachrymal surgery.[9] Cocaine therefore is easy to administer (intentionally or inadvertently) to a horse, because little skill is needed to apply small amounts of the drug to mucous membranes in the mouth, nose or genitalia.

After administration, cocaine is distributed in the body following a linear two-compartment pharmacokinetics model.[10] The plasma half-life of cocaine varies from 31 to 82 min, with a mean of 38 min.[2]

Cocaine is metabolized by liver and plasma esterases to two water-soluble metabolites—benzoylecgonine and ecgonine methyl ester —that are excreted in the urine.[11] Benzoylecgonine, compared with ecgonine methyl ester, is excreted more slowly and can be detected in urine for 72 h following cocaine use.

Many authors have investigated the possible correlation between physiological or behavioral effects and the concentrations of cocaine in plasma, saliva and brain. In a study with human subjects[12] it was concluded that the short cycle of cocaine stimulation and euphoria followed by dysphoria and craving may be related to the bioavailability of cocaine at central effector sites, which presumably is related to circulating plasma drug levels. Similar results were obtained with mice[13] and rats.[14] In this paper a good correlation was observed between the locomotor stimulation and the concentration of cocaine in the brain and in the serum. A precise prediction of dose, serum concentration and effects or impairment cannot be made for cocaine from the measurement of urine drug concentrations owing to a lack of scientifically established relationships between these parameters.[15]

On the other hand, to the best of our knowledge the minimum dose of cocaine required to produce a measurable pharmacological effect in a horse is about 4 mg per horse.[16] This dose of cocaine gives rise to easily detectable urinary concentrations of cocaine metabolites.

Exposure to small amounts of cocaine has been shown to produce positive results for urine cocaine metabolites by immunoassay. Amounts less than recreational doses (25 mg) have been given to humans without psychopharmacological effect, while producing positive urine tests.[15] It is clear today that urine concentrations may bear little relationship to clinical effects. Such findings are of significance when interpreting analytical results in racehorse drug testing, because positive results potentially could be caused by unknowing or unintentional exposure to small, subeffective doses of cocaine. The possibility that the cocaine sometimes detected in tests may be a result of 'accidental contamination' needs to be addressed.

Although the present study is limited to cocaine, it is important to stress that, at least in the USA, the majority of cases of positive samples refer to the detection of residual, insignificant quantities of therapeutic agents or their metabolites.[16] In this context, it should be recalled that veterinary medical practice and appropriate horse handling sometimes call for the administration of therapeutic agents to animals in training. Unfortunately, medicines administered for legitimate therapeutic purposes during the period before races can give rise to positive results in tests aimed at controlling the use of substances that artificially improve the animal's performance. This question is important because the absence of established acceptable concentration thresholds does not allow a distinction to be made between an accidental positive result and a positive result stemming from the illegal administration of controlled substances. Lastly, preoccupation with producing an accidental positive result could interfere with the legitimate therapy of racehorses, raising ethical questions such as therapeutic criteria and the well-being of the horses. The solution to this problem depends on the determination of the plasma and/or urinary concentrations of the active substance or its metabolites, which would not be linked to the pharmacological effect on the performance of the animal. There being no effect on performance, the presence of subliminal concentrations would be no reason for the disqualification of the animal.

We determined the maximal ineffective dose of cocaine on spontaneous locomotor activity of horses and examined the correlation between the administration of this dose and the urinary concentrations obtained at different times after drug injection. The main objective was to establish the maximum concentration threshold for this substance, in urine samples, that could not be associated with the artificial stimulation of performance.

EXPERIMENTAL

Animals

The study was conducted in 12 English thoroughbred mares from the herd of the Faculdade de Ciências Agrárias e Veterinárias of Jaboticabal, weighing between 400 and 550 kg. The animals were kept on pasture and offered additional mineral salts, *Cynodon* sp. hay *ad libitum* and pelletized commercial feed twice a day to give a total of 6 kg per day. The mares were weighed and sprayed every month with a pyrethroid insecticide (Butox: Químio SA) at the recommended dilution and wormed with 1% ivermectin (Ivomec: Merck Sharp and Dohme). The

experimental protocol was approved by the university's Institutional Animal Care and Use Committee.

Another five horses belonging to the experimental herd of the University of Kentucky were used to study the elimination of cocaine.

Drugs

Cocaine (cocaine hydrochloride; Merck, Darmstadt) was injected i.v. in doses of 0.02, 0.03, 0.04, 0.08 and 0.12 mg kg^{-1} diluted immediately before administration in 5 ml of sterile saline.

Construction of the behavioral stalls

Two behavioral stalls were constructed, equipped with juxtaposed photoelectric sensors installed at a height of 45 cm that emitted an infrared beam. Each time the beam was interrupted, a pulse was generated. The number of pulses was counted at 5-min intervals and stored in a data logger (Campbell Scientific Inc., Logan, UT) connected to a microcomputer for later analysis of spontaneous locomotor activity (SLA).

The stalls prevented the animals from seeing the outer environment and were equipped with two exhaust fans that, in addition to preventing the accumulation of gases, produced a white noise preventing the animals from hearing noises outside. Between the stalls there was a room with the controlling equipment. The room had two smoked-glass windows that permitted undetected observation of the animals' behavior when the lights were turned off. The configuration of the behavioral stalls for the quantification of horse SLA has been described in detail elsewhere.[17]

Experimental procedures

The animals were placed in the behavioral stalls the afternoon before the experiments, in order to acclimatize to the new environment. At 07.00 h they received feed and hay. At 09.00 h the sensors were turned on and cocaine or saline (control) was administered i.v. 45 min later (time zero). The animals were observed continuously by members of the research team over a period of 8 h from the time of drug administration.

At the end of this period, the sensors were turned off and the data were transferred to a 1.44-Mbyte floppy disk for later analysis. The stalls then were cleaned and new animals were placed there for experiments on the subsequent day. A 7-day interval elapsed between doses for each animal.

Data analysis

The data logger recorded the number of interruptions of each light beam in 5-min intervals. The values were summed and the mean for each time interval was calculated. The results of SLA were calculated as the number of interruptions per minute during the time interval between the last count and the next count. For example, SLA attributed to the 5-min time refers to the number of interruptions that occurred between time zero and 5 min, divided by the number of minutes (in this case, 5 min). Similarly, the SLA for the 90-min time refers to the number of interruptions that occurred between 75 min and 90 min divided by the number of minutes (in this case, 15 min).

Data were analyzed for statistical significance using the PROC GLM procedure of the SAS v. 6.11 computer software,[18] according to the following mathematical model

$$Y_{ijkl} = \mu + D_i + A_{ij} + T_k + TD_{ik} + e_{ijkl}$$

where μ = overall mean, D_i = effect of the ith cocaine dose (D_i = 0.02, 0.03, 0.04, 0.08 and 0.12 mg kg^{-1}), A_{ij} = nested effect of the ith dose within the jth animal, T_k = effect of the kth time (T_k = −30, −15, 0, 5, 10, ..., 480), TD_{ik} = effect of the interaction between the ith dose and the kth time and e_{ijkl} = random error, assumed to be normal and randomly distributed. All effects considered in the model are fixed. Specific statistical analyses also were performed for each dose and for each time. The mean values of dose and time effects were compared by Tukey's test ($P < 0.05$).

Determination of the dose–response curve

The dose–response curve was obtained based on the determination of the area between the curve of the control and that for each dose (area under the curve); a regression graph subsequently was elaborated.

Chemical Determinations

After determining the highest no-effect dose (HNED) for cocaine, five horses belonging to the experimental herd of the University of Kentucky were used to study the elimination of cocaine. For this propose, urine samples were collected before administration of the HNED (time zero) and at 1, 2, 4, 6, 8 and 24 h after HNED injection. These samples were taken with a flexible urethral catheter and stored at −20 °C. After each collection the urinary bladder was emptied completely and the biological material was analyzed to determine the concentrations of cocaine, norcocaine, benzoylecgonine and ecgonine methyl ester by means of liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS: Hewlett-Packard model 1050 HPLC instrument interfaced with a Micromass Quattro-II mass spectrometer operated in ESI-positive mode, Beverly, MA, USA).

RESULTS AND DISCUSSION

As shown in Fig. 1A, doses of 0.12 and 0.08 mg kg^{-1} cocaine induced effects characterized by an evident increase of SLA between the intervals of 5 and 20 min, which gradually decreased thereafter to the basal level. A significant difference was noted for these doses ($P < 0.05$) at 5, 10, 15, 20, 25, 30 and 45 min compared with the results obtained for the control group. Upon administration of a dose of 0.04 mg kg^{-1}, a considerable increase was noted in SLA 5 min after injection of the drug. Subsequently the SLA normalized, returning to basal levels within 20 min. Besides showing an increase of SLA, an evaluation of behavior changes revealed irritability and muscle tremors. The animals defecated within 3–10 minutes after administration of the substance and showed stereotypical movements, including bobbing of the head up and down, pawing at the ground, looking alert and showing uneasiness.

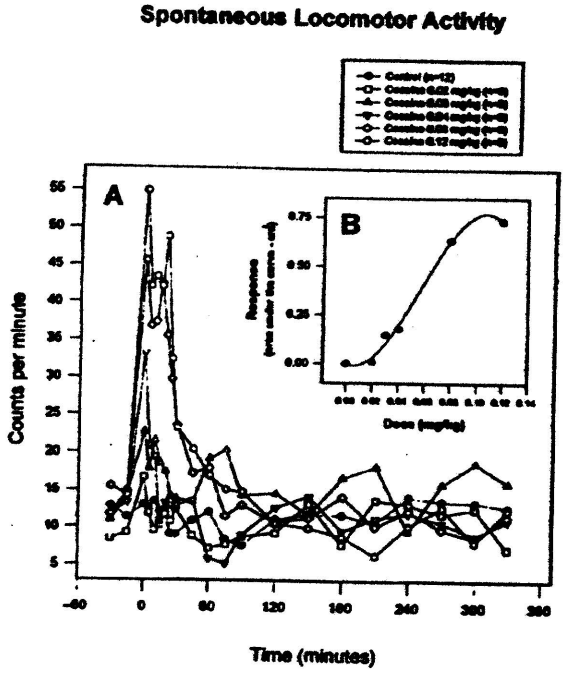

Figure 1. (A) Counts per minute refer.to the spontaneous locomotor activity of horses after the administration of increasing doses of cocaine (0.02, 0.03, 0.04, 0.08 and 0.12 mg kg $^{-1}$) or saline (control). (B) Cocaine dose–response curve, referring to its effect on the spontaneous locomotor activity of thoroughbred mares.

The animals neighed repeatedly during the first 5 min after injection. These changes were of quite short duration and the animals displayed normal behavior within a period of 15–20 min after administration of the substance. When administering cocaine at a dose of 0.03 mg kg $^{-1}$, no statistically significant difference was noted when the mean value of the SLA for this group was compared with that of the control group; however, a behavioral change was noted in 4/8 animals tested during the first 5 min after intravenous administration of the cocaine. These changes were the same as those observed in animals that received higher doses, consisting of head tossing and alert looks; three animals neighed and two animals defecated after the injection. Upon testing the dose of 0.02 mg kg $^{-1}$ cocaine no changes in SLA were noted when comparing the different groups during the intervals, or when comparing the different intervals within each group. Figure 1A also shows that with this dose the SLA remained similar to that observed in the control group, showing no behavioral changes.

Figure 1B shows the dose–response curve for cocaine on the SLA of horses. As can be seen, the curve that best fits the data appears to be sigmoidal, displaying an increase in pharmacological effect up to a dose of 0.12 mg kg $^{-1}$, which appears to be very close to that producing the maximum effect.

Based on the foregoing comments, the HNED for cocaine in horses was established at 0.02 mg kg $^{-1}$. Other researchers[3] performed tests to determine the performance of horses during exercise. The results showed that after administering cocaine intravenously at a dose of 0.11 mg kg $^{-1}$ no significant difference was noted when compared with the control group that received saline only. At a dose of 0.44 mg kg $^{-1}$, however, a significant

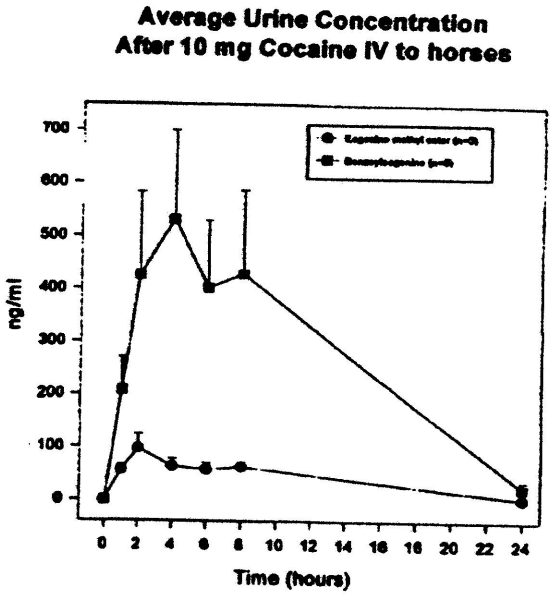

Figure 2. Kinetics of excretion of cocaine metabolites after intravenous administration of a single dose of 10 mg of cocaine to five thoroughbred mares. The vertical bars indicate the standard error of the means.

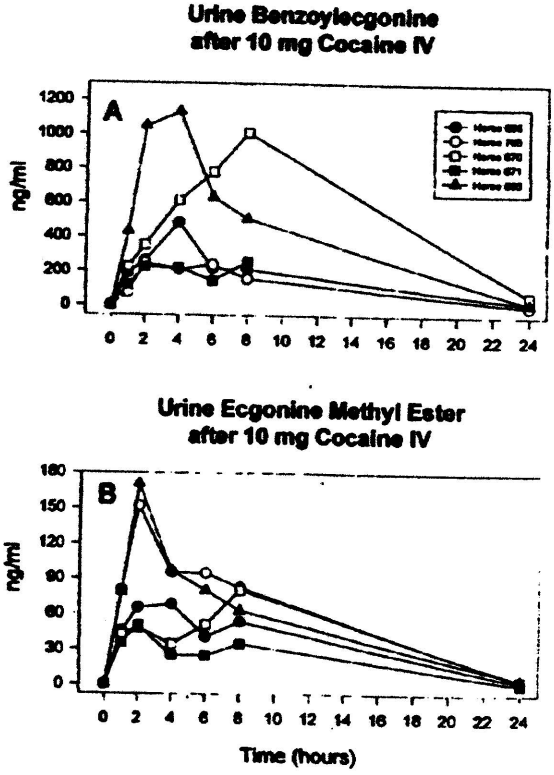

Figure 3. (A) Benzoylecgonine concentrations in the urine of five thoroughbred horses after intravenous injection of 10 mg of cocaine. (B) Ecgonine methyl ester concentrations in the urine of five thoroughbred horses after intravenous injection of 10 mg of cocaine.

increase in performance of the animals tested was noted. Meanwhile, our studies showed that there was a significant

increase in SLA already at a dose of 0.04 mg kg $^{-1}$. Higher doses (0.08 and 0.12 mg kg $^{-1}$) caused the animals to become quite restless, with an acute increase of SLA and profound behavioral disturbances. Small doses of cocaine primarily lead to the stimulation of euphoria and the potentiation of alertness and violent behavior, whereas moderate doses intensify euphoria.[2] The adrenergic stimulation becomes quite evident and leads to the manifestation of symptoms similar to those described for hyperthyroidism, which include increase of psychomotor activity, greater physical energy, tachycardia, muscle tremors and hyperthermia.[19] Behavioral studies with mice showed that the administration of cocaine in doses of 0.5–5 mg kg $^{-1}$ causes substantial changes, including an increase in aggressiveness.[20] Another study[14] stated that rats dosed with 35 mg kg $^{-1}$ showed ataxia and rotation of the head 6–10 min after drug injection. Doses of 0.06 and 0.07 mg kg $^{-1}$ cocaine induced a stimulant effect in rats, similar to that observed with the administration of amphetamines.[21] Similar effects such as head tossing, constant neighing and pawing at the ground were observed in our experiments frequently during the first minutes after cocaine administration: when using larger doses, two animals were observed compulsively biting the door of the behavior chamber (crib biting). These changes were observed in the animals tested with doses of 0.12, 0.08 and 0.04 mg kg $^{-1}$. At a dose of 0.3 mg kg $^{-1}$ this behavior was very much subdued for the reasons discussed above.

It was proved that, in human subjects, the concentrations of urinary cocaine are highest at 1–2 h and then substantially lower at 24 h after intranasal[22] or intravenous[23] administration. Figure 2 shows that the results obtained are consistent with those of the latter reports, i.e. the levels of benzoylecgonine reached a maximum value at 4 h after cocaine administration whereas ecgonine methyl ester reached a peak 2 h after injection. The concentrations of both metabolites were close to zero after 24 h. Cocaine and norcocaine concentrations remained consistently below the level of detection. On the other hand, Fig. 3 shows that the patterns of elimination for the two major cocaine metabolites can vary considerably, depending on the individual, making it difficult to establish a general elimination pattern.

It was concluded that for mares of the English thoroughbred breed the HNED of cocaine, determined in accordance with its capability of inducing an increase of SLA in a behavior chamber, is 0.02 mg kg $^{-1}$ when administered intravenously. The maximum admissible limit for the concentration of cocaine and/or its metabolites in horse urine is difficult to established unequivocally because of extensive individual variation in the elimination pattern observed in the horses, which can be inferred by the large standard error of the means obtained with the data.

Acknowledgements

This research was supported by grants funded by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil.

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