Appendix D

A Review of Methodologies Used to Determine the Highest No–Effect Doses (HNEDs) for Medications in Horses

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SUMMARY

We have described sensitive and reliable experimental methods that have been used to measure dose-related responses to various agents in horses. These methods can detect pharmacological responses to very small doses of medications in horses. The objective of this review was to describe the methods used to determine the highest no-effect dose (HNED) of drugs in racing horses. The HNEDs can then be administered to horses, and the concentrations of the drug/metabolite in the plasma/urine can be measured. The highest concentration of drug/metabolite could be used as the no-effect threshold (NET) for regulatory purposes. Although not all of the described methods have been used to determine HNEDs, the methods are sufficiently sensitive to determine the minimum doses likely to produce a pharmacological response.

INTRODUCTION

Within the past 15 years, various methods for quantitating the pharmacological effects of different medications in horses have been developed. All of the methods described below are sufficiently sensitive to detect the onset of pharmacological effects.

There are, of course, an infinite number of drug doses for which there is no pharmacologic effect. One of the purposes of this paper is to review previous studies that quantitated pharmacologic effects of

different drugs to determine the highest dose of drug for which there was no detectable pharmacologic effect. We have defined this dose as the highest no-effect dose (HNED). By definition, the HNED is "the highest drug dose for which there is no possibility of the horse having been pharmacologically influenced by the drug during a race." Once the HNED is established for a drug, that drug dosage can be administered, and the plasma/urinary concentrations of the drug/metabolite can be measured over a period of time after drug administration. The highest plasma/urinary concentration measured is then the no-effect threshold (NET). The NET is defined as "the highest plasma or urinary concentration of a drug or specified metabolite following HNED administration." Concentrations of drug below the NET found in post-race samples could, therefore, be chemically positive but, depending on the drug and the rule, could be forensically negative.

A broadly similar approach is standard in human forensic science, where positives for the "NIDA five" (Table 1) are not called if certain "cutoffs" (thresholds) are not exceeded. Application of the "thresholds" concept in veterinary science is simply an extension of an already well established practice in analysis of human drugs of abuse.

METHODS FOR ASSESSING PHARMACOLOGIC EFFECTS OF DRUGS

Step Counting Method

One of the simplest methods for quantitating the locomotor response to stimulant drugs in the horse is the step counting method first described by Tobin *et al* [1]. The horse was isolated in a box stall, and the left front leg was wrapped with white tape to assist the observer in counting the number of steps taken during a 2 minute period. Movement of the left leg not resulting in relocation of the left foot, such as pawing or scratching, was not counted.

Using this methodology, it was shown that 0.005 mg/kg of fentanyl injected intravenously significantly increased locomotion over baseline values. Incremental intravenous doses up to 0.02 mg/kg increased locomotion in a dose-dependent manner. However, there was no significant change in motor activity following intravenous administation of 0.001 mg/kg fentanyl. For this study, the relatively small dose of 0.001 mg/kg would be defined as the HNED, shown in Figure 1.

In the same study, apomorphine was administered intravenously, and motor activity was recorded. A dose of 6 mg of apomorphine produced no significant change in locomotion when compared to control values; whereas an intravenous dose of 18 mg produced a four-fold increase in activity, and a dose of 30 mg

Table 1 *National Institute of Drug Abuse (NIDA) thresholds :*

Drug	Screening Threshold	Confirmation Threshold
Amphetamines	1,000 ng/ml	500 ng/ml
Cocaine metabolites	300 ng/ml	150 ng/ml
Phencyclidine	25 ng/ml	25 ng/ml
Opiates	300 ng/ml	300 ng/ml
THC (marijuana)	50 ng/ml	15 ng/ml

Table 2 *HNEDs Determined by Different Methods:*

Drug	HNED	Method	Study
Fentanyl	0.001 mg/kg	Step Count	Tobin, 1979
Apomorphine	6.0 mg/kg	Step Count	Tobin, 1979
Morphine	0.3 mg/kg	Step Count	Combie, 1979
Pentazocine	0.5 mg/kg	Step Count	Combie, 1979
Hydromorphone	ND	Step Count	Combie, 1979
Meperidine	1.0 mg/kg	Step Count	Combie, 1979
Anileridine	0.05 mg/kg	Step Count	Combie, 1979
Methadone	ND	Step Count	Combie, 1979
Ethylketazocine	ND	Step Count, RR, HWRL, HR,Temp	Kamerling, 1986
Detomidine	0.01 mg/kg	STRL	Kamerling, 1988
	ND	Head Ptosis	
Procaine	5.0 mg	HWRL	Harkins, 1994
Bupivacaine	0.25 mg	HWRL	Harkins, 1994
Cocaine	45.0 mg	HWRL	Harkins, 1994
Acepromazine	0.004 mg/kg	Penile Pro	Ballard, 1982
	0.004 mg/kg	VIC	Ballard, 1982
	0.01 mg/kg	Resp Rate	Tobin, 1979
	0.00005 mg/kg	Hematocrit	Wood, 1992
Etorphin	<0.0002 mg/kg	Step Count	Combie, 1979
Methylphenidate	0.1 mg/kg	VIC	Shults, 1981
Reserpine	ND	VIC	Shults, 1981

ND= Not Determined

Figure 1 *Effect of fentanyl on locomotor activity in horses. The average counts/12-min period following saline injection is shown b ythe straight line near the bottom of the graph:*

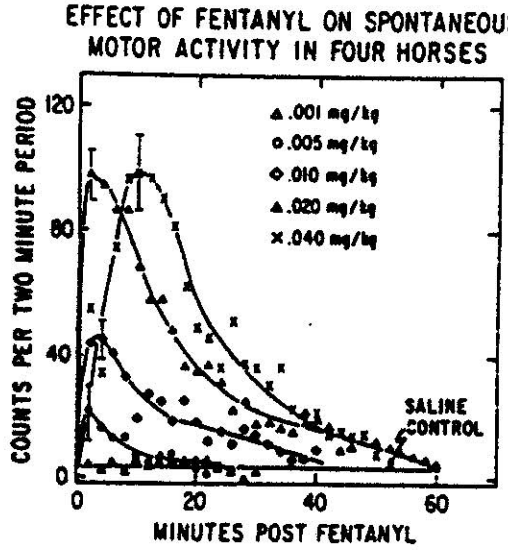
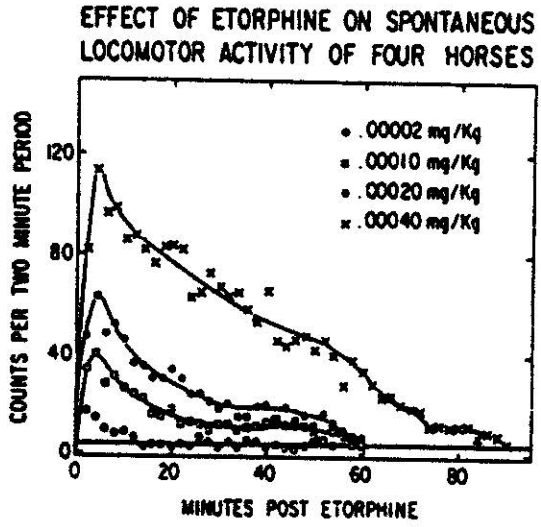


Figure 2 *Effect of etorphine on spontaneous locomotor activity in horses. The straight line at 4 counts/2-min period represents the average response to saline:*



produced a very sharp increase in locomotion. Based on these studies, a dose of 6 mg apomorphine would be defined as the HNED.

In a study of the effects of morphine, Combie et al 2 used similar methodology to show that locomotor activity was significantly increased following 0.6 mg/kg of morphine and increased in a dose-dependent manner through doses of 2.4 mg/kg. However, there was no significant effect on activity following doses of 0.1 and 0.3 mg/kg. The HNED from this study would be 0.3 mg/kg. Similar studies have assessed the effect on locomotor activity of other drugs including pentazocine, hydromorphone, meperidine, anileridine, methadone 2, and ethylketazocine 3.

The most potent narcotic available, etorphine, produced dose-response curves similar to those obtained from fentanyl, particularly in the low and intermediate dose ranges. Following a dose of 0.00002 mg/kg etorphine, there was no statistically significant effect; however, there was an increase in locomotor activity as may be seen in Figure 2.

Locomotor Chamber

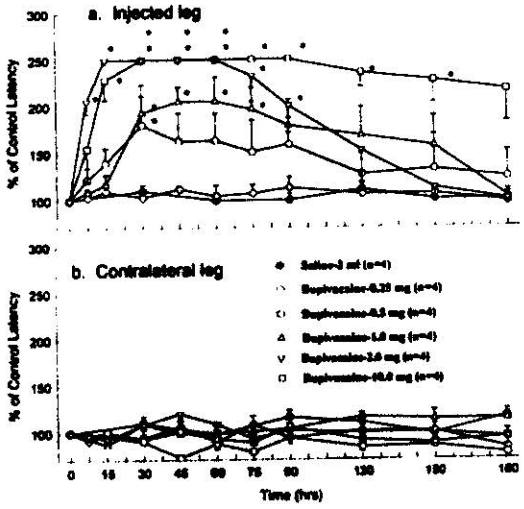
Kamerling *et al* 4 measured spontaneous motor activity in a box stall fitted with photoelectric counters. Following detomidine injection, doses of 0.01-0.04 mg/kg caused a significant decrease in motor activity. Therefore, all doses were above the HNED for detomidine.

Hoof Withdrawal Reflex Latency

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 5,6. 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 sec 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.

After administration of doses of 10.0, 20.0, and 40.0 mg procaine HCl, there was a significant difference between control and procaine values up to 30 min after injection of the anesthetic. However, there was no statistically significant local anesthetic effect

Figure 3 *Percent increase above control HWRL values following injection of incremental doses of bupivacaine. (repreduced with permission of the Equine Veterinary Journal):*



following injections of 2.5 and 5.0 mg procaine HCl[6]. The HNED for procaine was 5.0 mg/kg.

After administration of doses of 0.5, 1.0, 2.0, and 10.0 mg bupivacaine HCl, there was a dose-dependent local anesthetic response. Following doses of 0.25 mg, there was no significant difference between saline controls and bupivacaine values at any time post-injection. The HNED for bupivacaine was 0.25 mg/site (Figure 3). In a parallel study, the HNED for a significant local anesthetic effect of cocaine was 45 mg/site.

Skin Twitch Reflex Latency

In a method similar to that described under HWRL, the light/heat beam was focused on the skin of the withers, and the time required to elicit a skin twitch from the horse was measured. Kamerling *et al*[4] demonstrated that doses of 0.02-0.04 mg/kg of detomidine caused significant prolongation of the skin twitch reflex latency (STRL). However, for a dose of 0.01 mg/kg detomidine, there was no significant difference between STRL for control and treatment values. For this study, the HNED for detomidine was 0.01 mg/kg.

Penile Protrusion

Acepromazine is a tranquilizer widely used in equines to calm horses that become nervous when being transported or for restraint in a clinical situation. Small amounts of the tranquilizer could "take the edge off" a nervous horse before a race or show; however, this is clearly prohibited in racing and show horses.

One of the most readily apparent pharmacological responses of male horses to administration of phenothiazine tranquilizers is protrusion of the penis. A study by Ballard *et al*[7] determined that the extent of protrusion was dose-dependent and showed incremental increases in penile protrusion from 0.01 to 0.4 mg/kg. There was no significant effect on penile protrusion following a dose of only 0.004 mg/kg. For this study, the small dose of 0.004 mg/kg would be defined as the HNED.

Variable Interval Conditioning (VIC)

A specially designed stall was equipped with a light beam 10 cm above the feed bucket. When the head of the horse broke the light beam, the horse was "rewarded" with a small portion of oats falling into the bucket. Once the horses were conditioned to the apparatus, they tended to break the beam of light at a consistent and reproducible rate that was unique to each horse. The light-activated feeding console was used to measure the effects on the response activity of horses when treated with very small doses of drugs that produced no overt clinical signs. This method is especially sensitive to drug effects on the central nervous system[8].

Variable interval conditioning was used to assess the effect of acepromazine on behavior[7]. Doses of 0.01 to 0.4 mg/kg decreased the normal rate of light beam-breaking activity 20% to 55% of control in a dose-dependent manner. Similar to the HNED determined by penile protrusion, a dose of 0.004 mg/kg produced no significant change in light beam-breaking behavior.

In a study of the effects of methylphenidate (Ritalin^{R}) on behavior, Shults *et al* [9] administered incremental doses of 0.025 to 2.5 mg/kg of the drug and determined that doses of 0.25-1.0 mg/kg significantly increased the normal rate of light beam-breaking activity up to 600% of control values. Small doses of 0.025-0.1 mg/kg did not significantly affect light beam-breaking activity. The HNED for this study was considered to be 0.1 mg/kg.

Although the HNED for reserpine has not been determined, the sensitivity of this model to the effects of certain central nervous system depressants was illustrated in a study of the tranquilizing effects

of reserpine[10]. Clinically, reserpine causes sweating, depression, ptosis of the upper eyelid, and diarrhea. In this study, all of the clinical signs had abated by 48-72 hr after treatment, and the horses were clinically normal by 3 days after dosing. However, the maximal decrease in light beam-breaking activity did not occur until three days after dosing, and in one horse, the maximal response took 5 days to develop. The depressed light beam-breaking activity did not return to control levels until 6-10 days after dosing, several days after the clinical signs of reserpine administration had ceased.

PHYSIOLOGIC VARIABLES

Pharmacologic effects of drugs have been detected by measuring physiologic variables such as heart rate (HR), respiratory rate (RR), hematocrit, and body temperature[3,7,11,12]. Tobin and Woods[12] measured respiratory rates of horses administered incremental doses of acepromazine and demonstrated a dose-dependent decrease in respiratory rate in horses treated with 0.04-0.4 mg/kg acepromazine. However, there was no significant difference between respiratory rates of horses treated with 0.01 mg/kg acepromazine and control values. In this study, the HNED for acepromazine was 0.01 mg/kg.

One of the most sensitive variables for the detection of acepromazine administration is the change in hematocrit following administration of that drug. A dose of 0.002 mg/kg reduced hematocrit by 10%, and a dose of 0.01 mg/kg reduced hematocrit 25% within 30 minutes of administration[7]. Although the HNED was not determined in this study, it demonstrated that hematocrit is affected by very small doses of the acepromazine. In a subsequent study, Wood *et al*[13] showed no effect in hematocrit following injection of 0.01 and 0.05 ug/kg; however, there was a significant decrease in hemaotcrit following injection of 0.1-10.0 ug/kg. In this study, the HNED for acepromazine was 0.05 ug/kg.

HEAD PTOSIS

Normal sleep posture and drug-induced tranquilization and sedation are consistently accompanied by relaxation of the neck musculature and drooping (ptosis) of the head. Kamerling *et al*[4] placed horses in a specially designed stocks and measured the degree by which the head drooped by attaching one end of a wire to a standard halter worn by each animal and the other end to a spring-loaded reel. Doses of 0.01, 0.02, and 0.04 mg/kg of detomidine produced significant head ptosis within 10 minutes of administration. Although the HNED was not determined in this particular study, the sensitivity and reproducibility of the method illustrates its potential effectiveness for HNED determination of sedatives and tranquilizers used in horses.

CONCLUSIONS

1) For therapeutic medications, there is no logical need to detect non-effective residues of agents in the biological fluids of horses.

2) To determine a non-effective trace concentration, we must measure the maximum dose at which no pharmacological effect occurs.

3) This report reviews various research methods used to measure pharmacological effects. The effect of drugs on motor activity has been measured with step counting and locomotor chambers. The effect of drugs on pain perception has been measured with noxious heat stimuli. Mental activity has been measured with variable interval conditioning. This method is especially sensitive to drug effects on the central nervous system. Several physiologic variables are affected by drug administation. Some variables measured include heart and respiratory rates, body temperature, and hematocrit. Sedation and tranquilization have been correlated with the degree of head ptosis seen following administration of those drugs (Table 2).

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