

RADIOIMMUNOASSAY SCREENING FOR ETORPHINE IN RACING HORSES

W.E. Woods, T. Weckman, T. Wood, S-L Chang, J.W. Blake,
and T. Tobin

Kentucky Equine Drug Testing and Research Programs
Department of Veterinary Science
102 Animal Pathology Building
University of Kentucky
Lexington, KY 40546-0076

SUMMARY

A commercially available radioimmunoassay kit was used to screen for the presence of etorphine in post-race urines from horses racing in Kentucky. Most horse urines contained small amounts of materials which reacted positively in this immunoassay. These materials are apparently endogenous to the horse and were called apparent etorphine equivalents. The levels of these apparent etorphine equivalents in post-race urines from 70 horses were estimated. Their modal level averaged 0.1 ng/ml, the population distribution was log normal, and individual horses showed levels of up to 0.8 ng/ml.

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Correspondence should be addressed to Dr. Tobin.

Dosing horses with etorphine at rates from 1 µg/horse to 100 µg/horse yielded levels of exogenous etorphine equivalents in urine of from 1.0 ng to about 25 ng/ml. These levels of exogenous etorphine equivalents were easily distinguished from background etorphine equivalents values. The radioimmunoassay for etorphine is therefore sufficiently sensitive to allow good control of illegal use of etorphine in racing horses.

INTRODUCTION

Etorphine (4,5-epoxy-3-hydroxy-6-methoxy- α , 17-dimethyl- α -propyl-6, 14-ethenomorphinan-7-methanol) is a synthetic opiate derivative. It is synthesized from thebaine, a pharmacologically inactive opium alkaloid, and is structurally related to morphine. Etorphine is administered as the hydrochloride salt (M99®, oripavine), commonly in combination with a phenothiazine tranquilizer (Immobilon®-Large Animal Immobilon®; 2.45 mg etorphine HCl/ml with acepromazine; Small Animal Immobilon®; 0.074 mg etorphine HCl/ml with methotrimeprazine) (The Lancet, 1977). Because etorphine has been widely used in the capture of wild animals (such as elephants), it is sometimes referred to as "Elephant Juice." Under the name "Elephant Juice" it is widely known in racing circles as a potent and difficult to detect analgesic and stimulant.

Etorphine is a typical opiate narcotic analgesic characterized by extremely high potency (at least 10,000 times that of morphine), rapid onset, and short duration of action. The pharmacological actions of etorphine in man are similar to those of morphine, including the subjective effects and euphoria, but showing rapid onset and short duration (Jasinski, et al., 1975). In the horse, etorphine in

combination with acepromazine results in analgesia and sedation. Pharmacological responses to etorphine in the horse include tachycardia, depression of respiratory rate, spastic rigidity of the limbs and muscular tremors (Booth, McDonald, 1982). However, like other narcotic analgesics in the horse, low doses of etorphine result in stimulation of locomotor activity (Combie, et al., 1979).

In the horse, etorphine produces the classic locomotor response of the narcotic analgesics. After IV injection, horses rapidly increase their trotting activity, and if the dose is appropriate, (about 100 µg/horse) the locomotor response peaks at about 100 steps/2 minutes (Combie, et al., 1979). This response is qualitatively similar to the response seen with other narcotic analgesics. The principal difference between etorphine and the other narcotic analgesics is that the effects of etorphine occur after the injection of very small amounts of drug. For example, it takes only about 50 to 100 µg of etorphine/horse to produce a good locomotor effect, while it takes about 100 times this amount of fentanyl to produce the same effect (Combie, et al., 1979; Tobin, et al., 1979). Similarly, it takes about 100 to 300 mg of morphine to produce the same trotting response in a horse (Combie, et al., 1979). All other things being equal, these data indicate that etorphine is about 100 times more difficult than fentanyl to detect in a horse, and about 10,000 times more difficult than morphine. This is the reason for the suspected wide use of etorphine in racing horses, and the technical difficulty of detecting this drug on a routine basis.

More recently, a radioimmunoassay (RIA) for etorphine^a has become commercially available. In this report we demonstrate the ability of

this assay to detect etorphine (and/or etorphine metabolites) in the urine of horses dosed with amounts of etorphine likely to be used in racing horses.

MATERIALS AND METHODS

Etorphine RIA was performed using kits purchased from Karyon Technology Norwood, MA.^a The procedures followed were those supplied with the kits, with modifications as described by McDonald (McDonald, *et al.*, 1985). Standard curves, from 0.1 ng/ml to 10 ng/ml, were carried out in buffer and all levels of etorphine equivalents were calculated based on the standard curves.

In duplicate, 100 μ l of each standard, sample or control was added by pipette to 3 ml conical polystyrene test tubes. Standards were serial dilutions with assay buffer (phosphate buffered saline, pH 7.4, 0.1% bovine serum albumin and 0.1% sodium azide) of the etorphine stock standard (10 ng/ml) provided in the kit. Assay buffer was added to total count tubes (500 μ l), non-specific binding tubes (500 μ l), maximum binding tubes (400 μ l), standard tubes (300 μ l) and sample tubes (300 μ l). The buffer volume in the total count, non-specific binding, maximum binding, and standard tubes were reduced by 100 μ l if control urine was added to these tubes. The ³H-etorphine tracer stock solution from the kit was diluted with 5.5 ml of assay buffer per vial, and 100 μ l of diluted tracer was added to each tube. 100 μ l of Karyon antiserum (rabbit anti-etorphine antibody in assay buffer) was added to the maximum binding, standard, and sample tubes. All tubes were vortexed 3-4 seconds each. The tubes were incubated at 37° C in

^a Karyon Technology Inc., 333 Providence Highway, Norwood, MA 02062.

a water bath for 2 hours. After incubation, 800 μ l of ice-cold dextran coated charcoal suspension (0.25% dextran T-70 purchased from Sigma, St. Louis, MO, and 2.5% Norit A Charcoal from Fisher Scientific, Fairlawn, NJ) in assay buffer was added to all but the total count tubes. Assay buffer (800 μ l) was added to the total count tubes. All tubes were again vortexed 3-4 seconds each and then incubated for 30 min in a refrigerator. All the supernatant of each tube was decanted into 20 ml scintillation vials, and 10 ml of scintillation cocktail, 3A70b (Research Products International, Mount Prospect, IL), was added to each vial which was then vortexed. The samples were counted 10 min on a Beckman (Arlington Heights, IL) LS 3801 liquid scintillation counter.

Calculation of the data was by Logit-Log transformation of the dpm values for each tube:

$$L = \ln \left(\frac{B/B_0}{1 - B/B_0} \right)$$

where B_0 = the maximum binding dpm minus the non-specific binding dpm, and B = sample or standard dpm minus the non-specific binding dpm. Standard curves were constructed by plotting the logarithmic etorphine concentration of each standard versus the corresponding L value. The etorphine equivalent level for each sample was calculated from the standard curve for each run.

Post-race urines from track horses and urines from dosed research horses were tested for etorphine by RIA. Four research horses were dosed with M 99[®] (etorphine hydrochloride, D-M Pharmaceuticals, Rockville, MD) at 1 μ g, 9 μ g, 30 μ g and 100 μ g/horse, respectively. Urine samples were taken by bladder catheterization at hourly intervals for up to 8 hours post-dose, and at 24, 36 and 48 hours

post-dose. The urine samples were assayed directly without extractions. Dilutions of the dosed research horse urines, which were only necessary in horses receiving the 2 higher doses, were done with the control (pre-dose) urine from individual horses.

RESULTS

Fig. 1 shows standard curves for the etorphine assay performed in buffer (●-●) and with the addition of 100 μ l of equine urine (○-○). Although the horse from which the urine was obtained had no known exposure to etorphine, the standard curve in the presence of urine yielded higher values than those in buffer. This was a consistent response, and suggested that horse urine contains endogenous materials that react with the antibody. We elected to call these materials apparent etorphine equivalents. Based on these observations, we

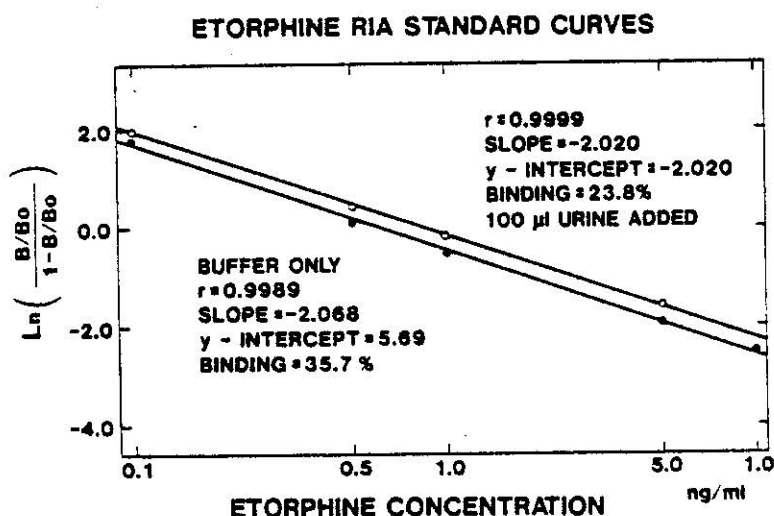


Fig. 1. Standard curves were constructed for etorphine in the presence of buffer and added urine. The solid circles (●-●) show the curve in the presence of buffer only, while the open circles (○-○) show the curve in the presence of 100 μ l added urine. Curves were fitted by least squares regression analysis.

carried out our standard assays in buffer, with no horse urine added, and computed all values obtained in horse urine on the basis of these standard curves.

Fig. 2 shows the levels of apparent etorphine equivalents in post-race urine samples collected in the state of Kentucky. Of the 69 post-race samples, 12 gave readings less than those of the control (buffer) samples, 15 yielded the median value of between 0.05 and 0.1 ng etorphine equivalents per ml and one had an etorphine equivalents figure of about 0.8 ng/ml. These values were log-normally distributed and appeared to form part of a single population (Fig. 2). There was an apparent tendency for horses from the research herd to yield higher values of etorphine equivalents than the race track horses. The reasons for these differences are not clear, but differences have been

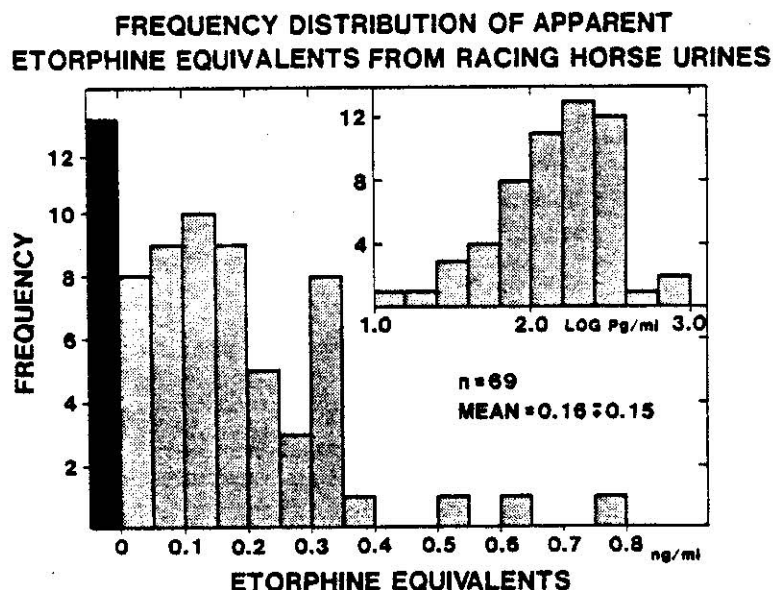


Fig. 2. Frequency distribution of endogenous etorphine equivalents in post-race urines. The vertical bars show the urinary levels of endogenous etorphine equivalents in post-race urines.

consistently observed over the last ten years in comparisons between post-race urine samples and samples from our research horses.

The possibility was considered that the apparent difference in etorphine equivalents between these two sets of samples might be related to differences in the specific gravity of our post-race urine samples. Because furosemide is a permitted medication in Kentucky, it was hypothesized that the diuretic effect of this drug might act to reduce the specific gravity of the urine in furosemide treated horses, and thus act to dilute out background etorphine equivalents. However, analysis of the specific gravity of these samples by refractometry showed no relationship between the apparent specific gravity of these samples and their content of etorphine equivalents (Fig. 3).

The radioimmunoassay test readily detected clinically effective doses of etorphine. Tobin and co-workers (Combie, et al., 1979) showed that doses of this drug in the order of 100 μg /horse produced a good locomotor response, such as might be useful in racing horses. This dose produced urinary levels of etorphine equivalents that peaked at about 25 ng/ml one hour after dosing and were still detectable at 24 hours after dosing (Fig. 4). Reducing the dose produced a corresponding reduction in the numbers of etorphine equivalents detected. For example, a dose of 1 μg /horse of etorphine produced changes in the numbers of etorphine equivalents in urine that were easily distinguishable from the background levels of etorphine equivalents. The test thus appears to detect levels of etorphine administration to horses of 1.0 μg /horse or more for at least four hours, more than sufficient for routine post-race drug screening.

RELATIONSHIP OF APPARENT ETORPHINE
LEVELS TO THE SPECIFIC GRAVITY OF URINE

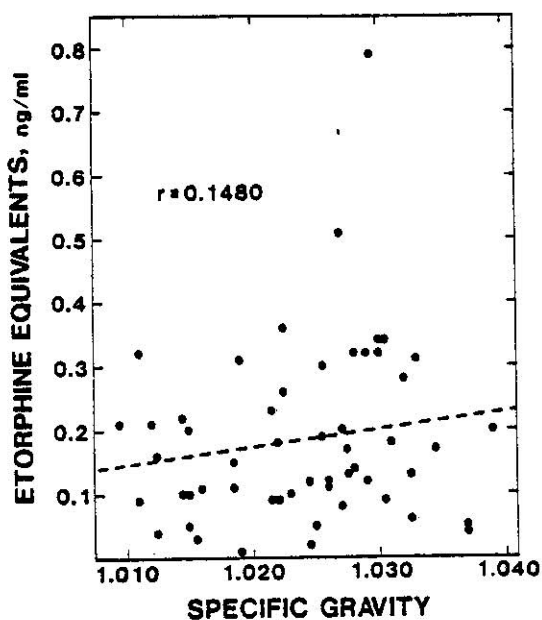


Fig. 3. Lack of correlation between urinary specific gravity and endogenous etorphine equivalents in post-race urines. The solid line shows the best fit to a plot of endogenous etorphine equivalents against urinary specific gravity. No apparent correlation was observed.

ETORPHINE EQUIVALENTS FROM DOSED HORSE URINE

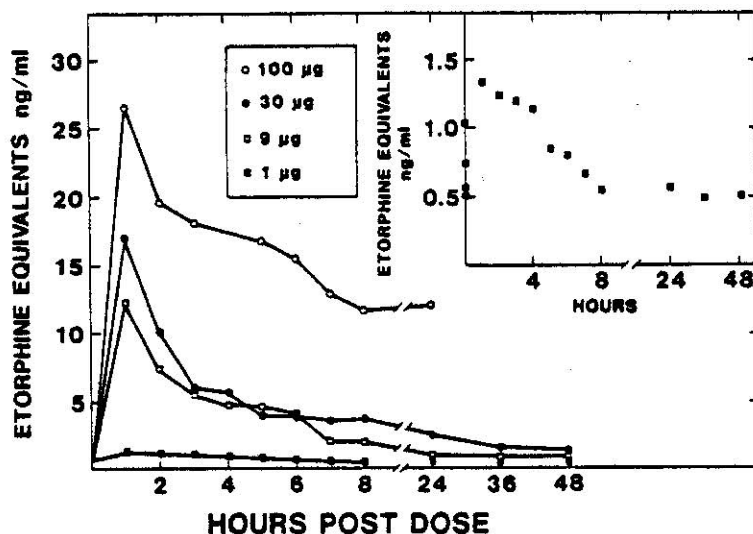


Fig. 4. The apparent levels of exogenous etorphine equivalents in horses after dosing with 100 $\mu\text{g}/\text{horse}$ (O-O), 30 $\mu\text{g}/\text{horse}$ (●-●), 9 $\mu\text{g}/\text{horse}$ (□-□), and 1 $\mu\text{g}/\text{horse}$ (■-■, insert). All values represent experimental points with one horse only.

DISCUSSION

These data show that post-race urines from horses racing in Kentucky contained materials which reacted in the etorphine immunoassay. Because these materials are apparently endogenous to the horse, they were called apparent etorphine equivalents. Because of the existence of this material, it was not possible to construct standard curves for etorphine in test systems containing urine. Therefore, the approach was taken of constructing our standard curves in buffer, which presumably contained no etorphine equivalents. Using these curves, the urinary content of etorphine equivalents in post-race urines of horses racing in Kentucky was estimated. It was assumed that these levels would represent background "noise" in the

assay, and that exogenous etorphine equivalents would be detected as signals in excess of this background noise.

Observation of the standard curve (Fig. 1) indicates that the likelihood of a background level of etorphine equivalents as high as 0.8 ng/ml could exclude the lower part of the curve (below 1 ng/ml) from use in detecting exogenous etorphine in urine. Due to the indirect nature of the competitive protein binding assay, the high end of the curve (above 5 ng/ml) results from very low dpm values (<200 dpm) and is therefore subject to a larger degree of counting error. It was found necessary to dilute urine samples with greater than 5 ng/ml of etorphine equivalents control urine in order to obtain a reliable estimate of drug levels.

Analysis of the data of Fig. 2 shows that the background levels of etorphine equivalents ("noise") in the post-race urines of horses racing in Kentucky was log-normally distributed. The modal level of etorphine equivalents was about 0.1 ng/ml, and the highest individual concentration was about 0.8 ng/ml. These levels tended to be somewhat lower than the levels of etorphine equivalents observed in horses from our research farm.

We considered the possibility that the one source of variation in urinary levels of apparent etorphine equivalents might be that horses racing in Kentucky may be treated with furosemide prior to racing. We therefore compared the urinary concentrations of etorphine equivalents to determine whether or not there was any correlation between the specific gravity of the urine sample and its content of etorphine equivalents. No evidence of any effect of specific gravity on urinary concentrations of background etorphine equivalents was observed,

suggesting that furosemide treatment does not have any significant effect on urinary content of etorphine equivalents.

When we treated horses with doses of etorphine of between 1 and 100 $\mu\text{g}/\text{horse}$, a clear effect on the levels of etorphine equivalents in urine were observed (Fig. 4). The horse dosed with 100 $\mu\text{g}/\text{horse}$ of etorphine showed urinary concentrations of etorphine equivalents of about 25 ng/ml at one hour after dosing, declining over the next 8 hours to about 10 ng/ml. By 24 hours after dosing the apparent level was 10 ng/ml, and the level was not followed further in this particular horse. Smaller doses of etorphine (30-90 $\mu\text{g}/\text{horse}$) gave rise to correspondingly lower levels of etorphine equivalents (Fig. 4).

When horses were dosed with 1.0 μg of etorphine, peak urinary levels of etorphine equivalents were 1.3 ng/ml, declining to background levels by eight hours after dosing (Fig 4, insert). If it is assumed that the background levels of endogenous etorphine are not more than 0.8 ng/ml, then administration of 1.0 μg of etorphine can be readily detected in post-race urines for about four hours after drug administration. Since the duration of action of etorphine after IV administration is short, and it is unlikely that this small dose of etorphine will have any pharmacological effect, it appears that this commercially available radioimmunoassay will detect any pharmacologically significant attempt to influence the performance of a race horse by the use of etorphine.

REFERENCES

Booth, N. and McDonald, L. (1982). Veterinary Pharm and Therap, 5th ed., Iowa State Press, p. 270-274.

Combie, J., Shults, T. and Tobin, T. (1979). The pharmacokinetics and behavioral effects of fentanyl and other narcotic analgesics in the horse. In 3rd Intl Symp on Eq Med Cont, p. 311-317, Lexington, Kentucky.

Jasinski, D., Griffith, D.R. and Carr, C.B. (1975). Etorphine in man, subjective effects and suppression of morphine abstinence. Clin Pharm and Therap, 17:267-276.

The Lancet (editorial staff). (1977). Immobilon: Curiously Strong. July 23 Issue, p. 178.

McDonald, J.L., Weidenbach, P., Merchant, S., Kalita, S.J., Calvo, P.P. and Duer, W.C. (1985). Analyses for etorphine in horse urine. Submitted for publication, Am J Vet Res.

Tobin, T., Combie, J., Shults, T., Dougherty, J. (1979). The pharmacology of narcotic analgesics in the horse. III. Characteristics of the locomotor effects of fentanyl and apomorphine. J Eq Med Surg, 3:284-288.

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