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Radioimmunoassay Screening for Etorphine in Racehorses

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Abstract

A commercially available radioimmunoassay kit was used to screen for etorphine in post-race urines from horses racing in Kentucky. Most horse urines contained small amounts of materials that reacted positively in this immunoassay. These materials are apparently endogenous in the horse. The levels of these endogenous etorphine equivalents in post-race urines from 69 horses were estimated. Their modal level was approximately 0.1 ng/ml, the population distribution was log-normal, and individual horses showed up to 0.8 ng/ml.

Dosing horses with etorphine (from 100 μg down to 1 μg per horse) yielded etorphine equivalents in urine from 1 to 25 ng/ml, which were easily distinguished from endogenous background values. The radioimmunoassay for etorphine is thus sensitive enough to allow good control of the use of etorphine in racehorses.

Introduction

Etorphine [6,7,8,14-tetrahydro-7α-(1-hydroxy-1-methylbutyl)-6,14-*endo*-ethenooripavine] 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 alone (M99) or, commonly, in combination with a phenothiazine tranquilizer (Large Animal Immobilon: 2.45 mg/ml etorphine HCl with acepromazine; Small Animal Immobilon: 0.074 mg/ml etorphine HCl with methotrimeprazine.[1] Because etorphine has been widely used for capturing 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 opioid analgesic characterized by extremely high potency (10000 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.[2] In the horse etorphine, in combination with acepromazine, produces analgesia and sedation. Pharmacological responses to etorphine in the horse include tachycardia, depression of respiratory rate, spastic rigidity of the limbs, and muscular tremors.[3]

However, like other narcotic analgesics in the horse, low doses of etorphine stimulate locomotor activity.[4] After IV injection horses rapidly increase their trotting activity and if the dose is appropriate (approximately 100 μg/horse) the locomotor response peaks at approximately 100 steps per 2 minutes.[4] This response is qualitatively similar to that seen with other narcotic analgesics, the main difference being that the effects of etorphine occur after injection of very small amounts. For example, only 50 to 100 μg of etorphine per horse is required to produce a good locomotor effect compared with approximately 100 times this amount of fentanyl or 100 to 300 mg of morphine.[4–5] Other things being equal, this data indicates that etorphine should be approximately 100 times more difficult than fentanyl to detect in a horse and nearly 10000 times more difficult than morphine. This explains the suspected wide use of etorphine in racehorses and why routine detection is hard.

Reagents for an etorphine radioimmunoassay (RIA) have recently become available commercially.

In this report we demonstrate the ability of this assay to detect etorphine (or etorphine metabolites) in the urine of horses dosed with amounts of etorphine likely to be used in racing.

Materials and Methods

Etorphine RIA was performed using kits purchased from Karyon Technology (Norwood, MA). The procedures followed were those supplied with the kits, with modifications as described by McDonald *et al.*[6] Standard curves were constructed from 0.1 ng/ml to 10 ng/ml in buffer and all levels of etorphine equivalents were calculated using the standard curves.

In duplicate, each standard, sample, or control (100 μl) was added by pipette to 3-ml conical polystyrene test tubes. Standards were serial dilutions with assay buffer (phosphate-buffered saline of pH 7.4 containing 0.1% bovine serum albumin and 0.1% sodium azide) of the etorphine stock standard (10 ng/ml) provided in the kits. Assay buffer was added to total-count tubes (500 μl), nonspecific-binding tubes (500 μl), maximum-binding tubes (400 μl), standard tubes (300 μl), and sample tubes (300 μl). The buffer volumes in the total-count, nonspecific-binding, maximum-binding, and standard tubes were reduced by 100 μl if control urine had been added to these tubes. The [^{3}H]etorphine-tracer stock solution from the kits was diluted with assay buffer (5.5 ml per vial) and diluted tracer (100 μl) was added to each tube. Karyon antiserum (rabbit etorphine antibody in assay buffer; 100 μl) was added to the maximum-binding, standard, and sample tubes. All tubes were vortexed (3–4 s), then incubated in a water bath (37°C; 2 h). After incubation ice-cold dextran-coated charcoal suspension (0.25% dextran T70 from Sigma, St. Louis, MO and 2.5% Norit 'A' Charcoal from Fisher Scientific, Fairlawn, NJ in assay buffer; 800 μl) was added to all but the total-count tubes. Assay buffer (800 μl) was added to the total-count tubes. All tubes were again vortexed (3–4 s), then incubated in a refrigerator (30 min). All the supernatant in each tube was decanted into 20-ml scintillation vials and scintillation cocktail (type 3A70b; Research Products International, Mount Prospect, IL; 10 ml) was added to each vial and vortexed. The samples were counted (10 min) using a liquid-scintillation counter (model LS 3801; Beckman Instruments, Arlington Heights, IL).

The fraction of labeled drug bound to the antibody was logit transformed using the expression

$$\text{logit}(B/B_0) = \ln\left(B/(B_0 - B)\right).$$

where B_0 is the maximum-binding d.p.m. minus the nonspecific-binding d.p.m., and B is the sample or standard d.p.m. minus the nonspecific-binding d.p.m. Standard curves were constructed by plotting the logarithmic etorphine concentration of each standard versus the corresponding logit 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 1, 9, 30, or 100 μg M99 (etorphine HCl; D–M Pharmaceuticals, Rockville, MD). Urine samples were taken by bladder catheter hourly for up to 8 hours after dosing, then at 24, 36, and 48 hours. The urine samples were assayed directly without extraction. Dilutions of the dosed research-horse urines (only necessary for etorphine-equivalent levels above 5 ng/ml) were prepared using the control (pre-dose) urines from these horses.

Results

Figure 1 shows standard curves for the etorphine assay performed in buffer alone and with the addition of equine urine (100 μl). Although the horse the urine was obtained from had had no known exposure to etorphine, the standard curve with urine had higher values than that for buffer alone. This was a consistent response and suggested that horse urine contains endogenous materials that react with the antibody. We elected to call these materials endogenous etorphine equivalents. Based on these observations we carried out our standard assays in buffer (with no horse urine added) and computed all horse-urine values from these standard curves.

Figure 2 shows the levels of endogenous etorphine equivalents in post-race urine samples collected in the state of Kentucky. Of the 69 post-race samples, 13 gave readings less than those of the control (buffer) samples, the median value was between 0.10 and 0.15 ng/ml, and the highest was approximately 0.8 ng/ml. These values were log-normally distributed and appeared to form part of a single population [Figure 2, insert]. Horses from the research herd tended

Figure 1 *Standard curves for etorphine in buffer alone (lower curve) and with 100 µl of added urine (upper curve) by RIA. Curves were fitted by least-squares regression analysis.*

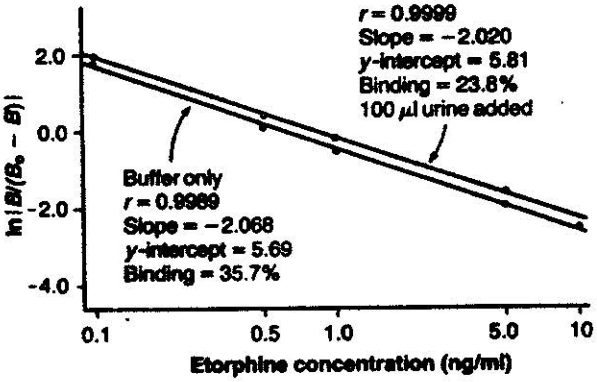
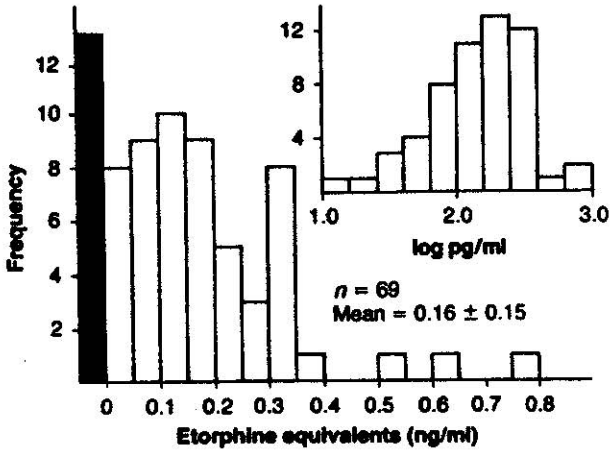


Figure 2 *Frequency distribution of endogenous etorphine equivalents in post-race urines*

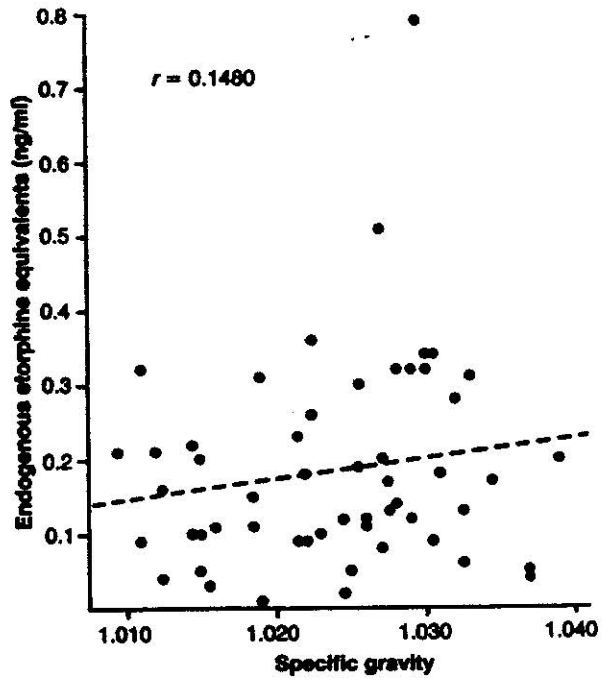


to have higher levels than the race-track horses. The reason for this is not clear but differences have been consistently observed over the last ten years in comparisons between post-race and research-horse urine samples.

Differences in the specific gravity of the urine samples were considered as one possible explanation for the apparent differences between the endogenous etorphine-equivalent concentrations of these two sets of samples. Because furosemide is a permitted medication in Kentucky it was hypothesized that the diuretic action of this drug might reduce the specific gravity of the urine in furosemide-treated horses and thus might also dilute the endogenous materials. However the specific gravity of these samples (as determined by refractometry) showed no relationship with their etorphine-equivalent content [Figure 3].

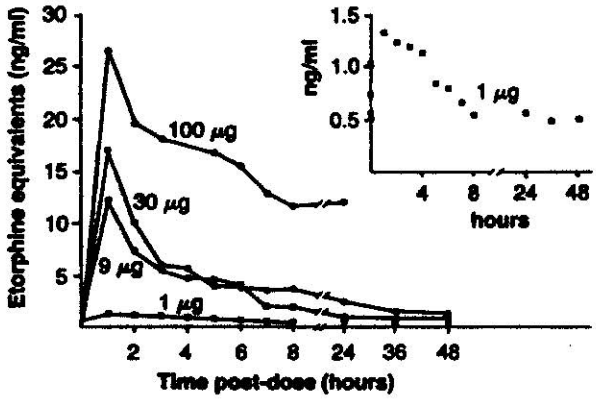
The RIA test readily detected clinically effective doses of etorphine. Tobin and co-workers[4] showed that doses of this drug in the order of 100 µg per horse produced a good locomotor response such as might be useful in racing. This dose produced urinary etorphine-equivalent levels that peaked at approximately 25 ng/ml 1 hour after dosing and were still detectable at 24 hours [Figure 4]. Reducing the dose produced a corresponding reduction in the etorphine equivalents detected. A dose of 1 µg of etorphine produced changes in the etorphine equivalents in urine that were easily distinguishable from endogenous

Figure 3 *Lack of correlation between urinary specific gravity and endogenous etorphine equivalents in post-race urines. (The line shows the best fit.)*



background. The test thus appears to detect etorphine doses of 1.0 µg or more for at least 4 hours—more than sufficient for routine post-race drug screening.

Figure 4 *Etorphine equivalents in urine from horses dosed with 100, 30, 9, and (insert) 1 μg etorphine hydrochloride. (Each experimental point is for one horse only.)*



Discussion

This data showed that post-race urines from horses racing in Kentucky contained materials which reacted in the etorphine immunoassay. Because of these materials standard curves could not be constructed for etorphine in urine. Therefore our standard curves were constructed for etorphine in buffer which presumably contained no etorphine equivalents. Using these curves, the endogenous etorphine-equivalent content 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 exceeding this background noise.

Background endogenous equivalents as high as 0.8 ng/ml exclude the lower part of the standard curve (below 1 ng/ml) from use in detecting exogenous etorphine in urine [Figure 1]. Due to the nature of the antibody-binding assay the high end of the curve (above 5 ng/ml) results from very low counts (< 200 d.p.m.) and is therefore more subject to counting error. Urine samples with more than 5 ng/ml of etorphine equivalents had to be diluted with control urine to obtain reliable estimates of drug levels.

Analysis of the Figure 2 data showed that the background etorphine equivalents ('noise') in the post-race urines of horses racing in Kentucky were log-normally distributed. The mode was approximately 0.1 ng/ml; the highest individual concentration was approximately 0.8 ng/ml. These levels tended to be somewhat lower than the levels observed in horses from our research farm.

We hypothesized that one source of variation in the urinary endogenous levels might be that horses racing in Kentucky can be treated with furosemide before racing. To test this hypothesis we compared the urinary concentrations of endogenous etorphine equivalents with the urine-sample specific gravity. No evidence of any correlation between specific gravity and urinary etorphine-equivalent concentration was observed, suggesting that furosemide treatment does not have any significant effect on urinary endogenous etorphine-equivalent content.

When we treated horses with doses of etorphine (1–100 μg/horse) a clear effect on the etorphine-equivalent levels in urine was observed [Figure 4]. The horse dosed with 100 μg of etorphine showed a urinary etorphine-equivalent concentration of approximately 25 ng/ml 1 hour after dosing, declining over the next 8 hours to approximately 10 ng/ml. At 24 hours the level was still approximately 10 ng/ml but was not followed further. Smaller doses of etorphine resulted in correspondingly lower etorphine-equivalent levels [Figure 4].

For the horse dosed with 1.0 μg of etorphine, the peak urinary etorphine-equivalent level was 1.3 ng/ml, declining to background levels by 8 hours after dosing [Figure 4, insert]. Assuming a maximum endogenous etorphine-equivalent level of 0.8 ng/ml, doses of 1.0 μg of etorphine can be readily detected in post-race urines for approximately 4 hours. Since the duration of action of etorphine after IV administration is short, and it is unlikely that such a small dose will have any pharmacological effect, this commercial radioimmunoassay will detect any pharmacologically significant attempt to influence the performance of a racehorse with etorphine.

Acknowledgments

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Discussion

(Discussion follows the next paper, on a related topic.)