Reprinted from the AMERICAN JOURNAL OF VETERINARY RESEARCH, Vol. 49, No. 5, Pages 622-628.
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Radioimmunoassay for etorphine in horses with a ^{125}I analog of etorphine

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SUMMARY

To improve the sensitivity and specificity of screening for etorphine in horses, an ^{125}I-labeled etorphine analog was synthesized and an antibody to etorphine was raised in rabbits. A radioimmunoassay (RIA) for etorphine was developed, using these reagents.

Bound and free ^{125}I-labeled etorphine was separated by a double-antibody method that reduced interference from materials associated with equine urine. The ^{125}I-labeled etorphine binding was rarely > 250 pg of background etorphine equivalents/ml in raw urine and was 100 pg/ml in hydrolyzed urine. The ^{125}I-RIA was capable of detecting etorphine equivalents in urine above these background values.

Etorphine equivalents were detected in equine urine samples for about 7 days after 4 mares were dosed with 0.22 μg of etorphine/kg of body weight, IV. The stability of etorphine in urine from these mares was evaluated. Urine from these dosed mares was held in constant −20 C storage, and aliquots were repeatedly frozen and thawed. When analyzed for etorphine equivalents using an ^{125}I-RIA, etorphine and its metabolites in urine samples were stable for ≤ 38 days if continuously frozen and also were resistant to repeated freezing and thawing.

Etorphine (4,5-epoxy-3-hydroxy-6-methoxy-α,17-dimethyl-α-propyl-6,14-ethenomorphinan-7-methanol), a synthetic opiate derivative, is a typical morphine-like agonist narcotic analgesic characterized by high potency (at least 10,000 times the potency of morphine), rapid onset, and short duration of action. In human beings, pharmacologic actions of etorphine are similar to those of morphine, including subjective effects and euphoria, but differ in having rapid onset and short duration of action.[1]

In horses, etorphine in low doses (< 100 μg/horse) induced stereotypic locomotor activity (circling) similar to the response seen with other narcotic analgesics such as morphine and fentanyl.[2] In horses in a stall, only 50 to 100 μg of etorphine/horse induced a 10-fold increase from random locomotor activity vs 5 to 10 mg of fentanyl/horse to induce the same effect.[3] Approximately 100 to 300 mg of morphine induced the same amount of locomotor response in horses.[4]

Because etorphine induces locomotor stimulation at low doses, etorphine may be 100 times more difficult to detect than fentanyl and about 10,000 times more difficult to detect than morphine. This high potency, along with the locomotor-stimulation characteristic of narcotic analgesics in horses, makes etorphine a potential stimulant in racehorses. Other narcotic analgesics have been used widely as such. Use of these narcotic agents is illegal and has created a requirement for sensitive tests to detect these drugs in urine samples taken from horses after racing. Control of the illegal use of etorphine depends on the availability of rapid, sensitive, and inexpensive screening methods. The current method of choice is a radioimmunoassay (RIA).

A ^{3}H-labeled etorphine RIA has been developed to screen for etorphine in equine urine and has been used in a number of racing jurisdictions. However, equine urine contains materials that cause high-background values in immunoassays, which can cause problems in the screening of race samples. This ^{3}H-RIA has a relatively high nonspecific background value, and when used with urine samples, this background interferes with usefulness of ^{3}H-RIA to detect low etorphine concentrations.[4] The purpose of the study reported here was to describe a RIA for etorphine, that would allow detection of small amounts of etorphine in equine urine.

Materials and Methods

Preparation of succinyl etorphine—Etorphine-3-hemisuccinate was prepared by reacting succinic anhydride[a] with etorphine. Etorphine HCl[b] (25 mg) was suspended in 4 ml of tetrahydrofuran, to which 112 μl of 1*N* sodium hydroxide was added with stirring at room temperature (25 C, Fig 1). The suspension turned into solution after 10 minutes of stirring. Succinic anhydride (11 mg) was added, and the reaction was allowed to proceed at 25 C for 16 hours followed by refluxing

Received for publication Oct 27, 1986.

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Supported by a grant from the Kentucky State Racing Commission, Kentucky Harness Racing Commission, and the Kentucky Equine Drug Research Council.

Published as Kentucky Agricultural Experiment Station article No. 85-4-240 with the approval of the dean and director, College of Agriculture and Kentucky Agricultural Experiment Station. Publication No. 131 from the Kentucky Equine Drug Research and Testing Programs, Department of Veterinary Science and the Graduate Center for Toxicology, University of Kentucky, Lexington.

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[a] Sigma Chemical Co, St Louis, Mo.

[b] American Cyanamid Co, Princeton, NJ.

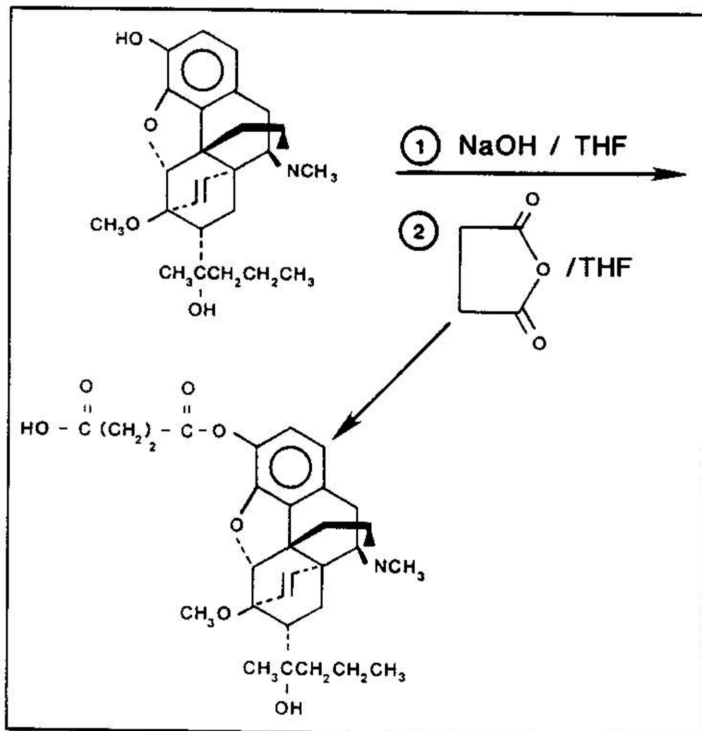

Fig 1—Synthesis of etorphine-3-hemisuccinate. Etorphine was dissolved in tetrahydrofuran (THF)/sodium hydroxide and was reacted with succinic anhydride.

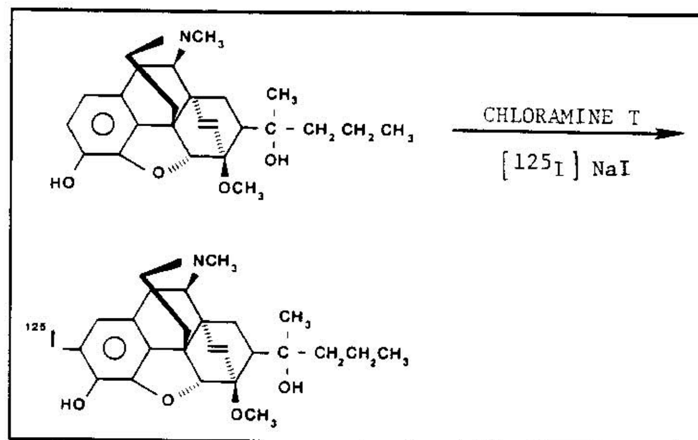

Fig 2—Iodination of etorphine. ^{125}I-labeled etorphine was prepared by reacting etorphine HCl with [^{125}I]sodium iodide in the presence of chloramine T.

for 1 hour. After the solvent was removed, the mixture was purified by preparative thin-layer chromatography on a silica gel G plate developed in chloroform/methanol (10:1), and 20 mg of etorphine-3-hemisuccinate was obtained. Addition of a succinyl group was confirmed by infrared and nuclear magnetic resonance analysis.

Etorphine antibody—Etorphine-3-hemisuccinate was conjugated to bovine serum albumin (BSA) as described for thromboxane B2.[5] Briefly, etorphine-3-hemisuccinate (10 mg) was dissolved in 0.6 ml of ethanol, and 5.4 ml of 0.02% sodium bicarbonate and 30 mg of BSA were added. 1-Ethyl-3-3-(3-dimethylaminopropyl) carbodimide[b] (50 mg) was added as a coupling agent, and the mixture was stirred for 16 hours at 4 C and was dialyzed against 1 L of saline solution for 48 hours with 3 changes of saline solution. About 1 mg of the conjugate in 1 ml of saline solution was emulsified with 1 ml of Freund complete adjuvant[c] and was given SC to each of 4 rabbits at multiple sites across the back. Booster injections were carried out at the same dose on a monthly basis. One week after each inoculation, heparinized blood was collected by venipuncture from the ear veins and was centrifuged at 3,000 × g for 15 minutes. Production of the antibody was monitored by evaluating the antibody's ability to bind with ^{125}I-labeled etorphine. Standard curves were used to determine the sensitivity of each antibody. Antibody was aliquoted and stored at −20 C.

Radioiodination of etorphine—Because etorphine possesses a phenolic group, radioiodination can be carried out directly (Fig 2). Etorphine HCl (1 µg) was dissolved in 50 µl of 0.5*M* sodium phosphate buffer (pH 7.5) to which [^{125}I]sodium iodide[d] (300 µCi) in 3 µl of 0.01*N* sodium hydroxide was added. Iodination was initiated by the addition of 25 µg of chloramine T[e] in 5 µl of 0.05*M* sodium phosphate buffer (pH 7.5) and was terminated after 30 seconds of reaction by addition of 250 µg of sodium metabisulfite in 50 µl of 0.05*M* sodium phosphate buffer (pH 7.5). The reaction mixture was extracted twice with 0.6 ml of ethyl acetate. The organic layer was evaporated to dryness with a stream of nitrogen. The residue was spotted on a silica gel G plate (2 × 20 cm) with chloroform/methanol/acetic acid (90:10:1) as the developing solvent. The location of ^{125}I-labeled etorphine derivative was identified by autoradiography. The band corresponding to labeled etorphine was scraped off the gel and was extracted with 1 ml of ethanol twice. After the gel was removed by centrifugation, labeled etorphine was stored at −20 C.

RIA of etorphine—The reaction mixture included 0.1 ml of sample or etorphine standard, 0.2 ml of diluted antibody, and 0.1 ml of ^{125}I-labeled etorphine (about 10,000 counts per minute). All standards, antibody, and labeled etorphine were diluted in RIA buffer (0.05*M* Tris-HCl, pH 7.5 containing 0.1% gelatin and 0.01% thimerosal[a]). All samples were run in duplicate in 10 × 75-mm glass tubes. The incubation was allowed for 90 minutes at 25 C. Separation of bound and free ^{125}I-labeled etorphine was achieved by 2 methods. In the first method, water (1 ml) and γ-globulin-coated charcoal (1% bovine γ-globulin,[a] 3% charcoal[a] in 200 µl of assay buffer) was pipetted into each tube after incubation. Tubes were vortexed and allowed to stand for 5 minutes at 25 C before centrifugation at 2,000 × g for 5 minutes. The supernatant (bound form) was pipetted into clean 10 × 75-mm glass tubes and was counted in a gamma counter.[f] When separation by a second antibody method was used, a 10-fold diluted normal rabbit serum (20 µl) and 50 µl (0.5 U) goat anti-rabbit γ-globulin serum[g] (2nd antibody) were added, and incubation was continued for another hour at 25 C. At the end of the 2nd incubation, 1 ml of cold 0.05*M* Tris-HCl (pH 7.5) was added to the tube, and tubes were placed in ice for 10 minutes before centrifugation at 2,000 × g for 15 minutes at 4 C. The supernatant was aspirated, and radioactivity in the precipitate (bound form) was quantified in a gamma counter. Sample concentrations were calculated, using a Spline function to fit the standard curve.[6,7]

RIA sensitivity—Evaluation of assay sensitivity was conducted by dosing 4 mares and monitoring detectable amounts of etorphine equivalents. The ^{125}I-RIA was conducted on nonhydrolyzed and hydrolyzed samples. Interassay precision was not evaluated because of the small number of samples (< 100 tubes) assayed in each RIA run.

[c] Calbiochem, Behring Diagnostics, Division of American Hoechst Corp, San Diego, Calif.
[d] Amersham Corp, Arlington Heights, Ill.
[e] Kodak Laboratory Chemical Co, Rochester, NY.
[f] LKB Instruments, Gaithersburg, Md.
[g] Calbiochem, Behring Diagnostics — Division of American Hoechst Corp, San Diego, Calif (1 U = amount that will precipitate the γ-globulin in 200 µl of 2% normal rabbit serum during 6 hours' incubation at 25 C).

Cross-reactivity study—A study of potential cross-reacting drugs was conducted to validate the specificity of the antibody developed. Varying concentration solutions of morphine, thebaine, codeine, diprenorphine HCl,[h] and carfentanil[i] were used as samples and were run simultaneously with etorphine standards.

Urine sample backgrounds—Equine urine samples often contain endogenous materials that cross-react with antibodies to varying degrees in RIA systems. Postrace urine samples from horses racing in Kentucky over a 7-month period were screened to determine amounts of cross-reacting materials that were likely to be detected by both RIA. Equine urine samples were tested, using ^{3}H-RIA[j] (n = 301) and ^{125}I-RIA (n = 101). In each case, nonhydrolyzed urine samples were examined. Population distributions of background material in urine samples were analyzed and fitted to various statistical functions. The function offering the best fit was selected, and the probability of yielding a background above certain values was determined.

Urine hydrolysis and extraction—Hydrolysis may be a method to reduce background cross-reactants in equine urine samples tested for etorphine in RIA.[k] Postrace urine samples from 26 horses racing in Kentucky were obtained. Each sample was aliquoted on receipt and was stored frozen at −20 C until analysis. Urine samples (1 ml) were hydrolyzed with 2 ml of β-glucuronidase[a] (*Patella vulgata*, 2,500 U) in 1*M* sodium acetate buffer (pH 5) for 3 hours at 60 C. Concentrated ammonium hydroxide was added to each tube to adjust the pH to 9. After hydrolysis, various organic solvent systems were tested for extraction. The organic layer was separated and evaporated to dryness with nitrogen, and the RIA was performed after dried residues were suspended in RIA buffer. For routine work, a mixture of petroleum ether/methylene chloride (2:1) was used for etorphine extraction. Raw urine samples without treatment were analyzed by the ^{125}I-RIA.

Sample stability—Aliquots of diluted postdose urine (high control) and predose urine (low control) from the 4 mares were stored continuously at −20 C and were analyzed at various intervals up to 38 days after initial freezing. The impact of freezing and thawing on the stability of etorphine equivalents in urine samples from the 4 mares also was monitored. Aliquots of 6 urine samples were frozen at −70 C and were thawed in a 25 C water bath. The freeze-thaw cycle was repeated up to 7 times before samples were analyzed using ^{125}I-RIA.

Mares—Etorphine administration experiments were conducted in 4 mature Thoroughbred and Standardbred mares (12 to 21 years old) weighing about 550 kg ($\bar{X}$ = 550.3 kg). These mares were healthy and not pregnant. Mares that had been used in previous studies had been dosed no closer than 6 weeks before inclusion into the present study. These mares were kept on pasture and were brought into box stalls 24 hours before drug administration. Etorphine (0.22 μg/kg of body weight) was administered as etorphine HCl[l] by rapid injection into the jugular vein. Urine samples were collected by direct urinary bladder catheterization immediately before dosing and at 1, 2, 4, 6, and 24 hours after dosing and each 24 hours thereafter for 10 days. These urine samples were aliquoted and stored frozen at −20 C.

Forensic study—In etorphine equivalent background studies, forensic urine samples (1 sample/horse, n = 402), were collected from horses after races under the supervision of the racing authority in charge at the individual racecourse. Urine samples were then delivered to the Kentucky Equine Drug Testing Program by a secure courier.

[h] M50-50, D-M Pharmaceuticals, Rockville, Md.
[i] Wildnil, Wildlife Laboratories, Fort Collins, Colo.
[j] Cambridge Medical Diagnostics Inc, Billerica, Mass.
[k] John McDonald, Illinois Racing Board Laboratory, Elgin, Ill: Personal communication, 1986.
[l] M-99 etorphine, D-M Pharmaceuticals, Rockville, Md.

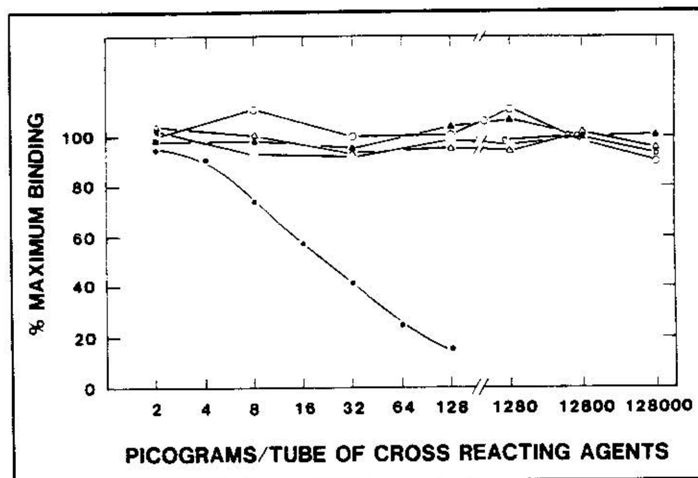

Fig 3—Specificity of etorphine antibody. Selected compounds at the indicated amounts (x axis) were incubated with etorphine antibody in the presence of ^{125}I-labeled etorphine. Inhibition of maximal binding by these compounds is indicated (y axis). ● = etorphine; △ = morphine; ▲ = thebaine; ■ = codeine; ○ = diprenorphine HCl; □ = carfentanil

Results

Separation of bound and free ^{125}I-labeled etorphine—Preliminary experiments with iodinated etorphine and our etorphine antibody revealed that high background values of nonspecific binding developed in the presence of equine urine. The charcoal method for removal of free ^{125}I-labeled etorphine gave unusually high background values in equine urine, which would have interfered with the accuracy of the test. Nonspecific background counts from the charcoal method resulted in 27.5% of total counts, whereas the use of a second antibody separation technique reduced the background counts to 1%. Therefore, we altered the separation method, and used a second antibody to precipitate the ^{125}I-labeled etorphine antibody complex. When the test was modified, background values were substantially reduced, and the test was sensitive down to about 2 pg of etorphine added to the test systems. In the ^{125}I-RIA, cross-reactivity with other drugs was minimal. Morphine, thebaine, codeine, diprenorphine HCl (an etorphine antagonist), and carfentanil did not induce marked displacement of etorphine from this antibody (Fig 3).

Background material in urine samples—Our experience with RIA of equine urine samples indicated that the limiting factor in the efficacy of these tests was not the sensitivity of the test, but rather the ability of background materials in equine urine to interfere with the test. Therefore, we used ^{3}H-RIA and ^{125}I-RIA to determine etorphine equivalents in 402 urine samples collected after races in Kentucky (Fig 4). Interfering background materials in the ^{125}I-RIA are about half of those seen in the ^{3}H-RIA (Fig 4). For example, only about 1% of background values > 250 pg/ml was likely to be detected in the ^{125}I-RIA, in contrast with about 638 pg/ml for 99% of the background material in the ^{3}H-RIA (Table 1).

Further reduction of background materials—Background values of 26 urine samples before and after hy-

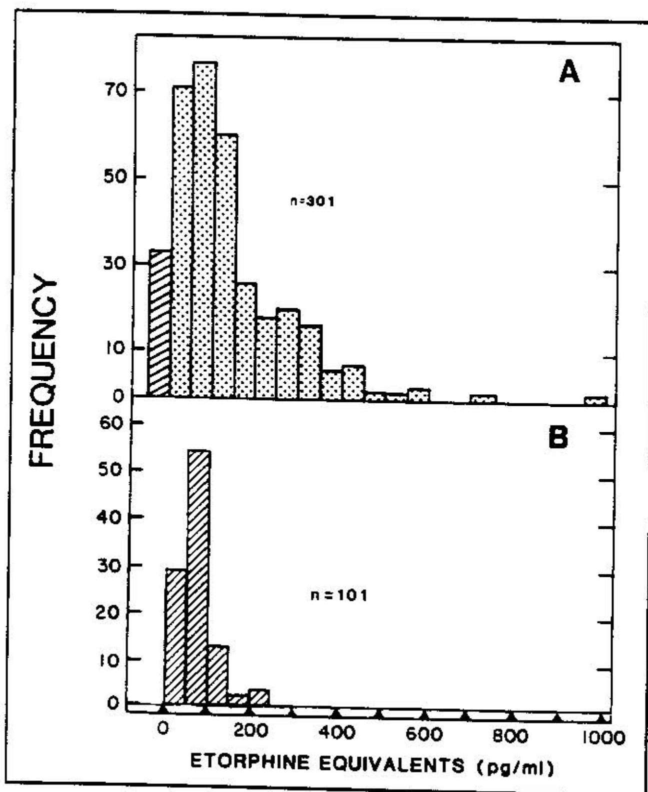

Fig 4—Etorphine equivalents of 402 horses racing in Kentucky.
A—etorphine equivalents as determined by the ^{3}H-labeled etorphine RIA in postrace urine of horses racing in Kentucky (n = 301).
B—etorphine equivalents in postrace urine samples, as determined by ^{125}I-labeled etorphine RIA (n = 101). All determinations were performed on nonhydrolyzed urine samples.

TABLE 1—Percentage of background values below indicated values in a radioimmunoassay (RIA)

	Value (pg/ml)			
RIA	< 95%	< 99%	< 99.9%	Mathematical model
^{3}H	...	250	500	Erland distribution
^{125}I	381	638	895	Log-normal distribution

The data of Figure 4 were analyzed, and the mathematical model giving the best fit to the curves was selected. From this model, percentages of population values likely to be found below the indicated apparent etorphine values were estimated.

drolysis were compared (Fig 5). Hydrolysis and extraction reduced the background value by 50%, in the case of ethyl acetate extraction, and even more in the case of petroleum ether extraction. However, recovery of etorphine into petroleum ether after a single extraction was low (Fig 6). Therefore, for routine work, a mixture of petroleum ether/methylene chloride (2:1) for etorphine extraction was used.

^{125}I-RIA sensitivity—To determine ^{125}I-RIA sensitivity, 4 mares were given etorphine at 0.22 μg/kg, and the urinary concentrations of etorphine equivalents were monitored. Etorphine was detectable in the urine of these mares at above background values for about 5 days (Fig 7). However, after 6 days, urinary amounts of etorphine equivalents were not distinguishable from background values. Background values in the ^{125}I-RIA in urine from mares maintained on pasture averaged about 100 pg/ml, and amounts ≤ 100 pg of etorphine equivalents/ml were not likely to be detectable in nonhydrolyzed postrace urine samples. Test sensitivity also was determined in hydro-

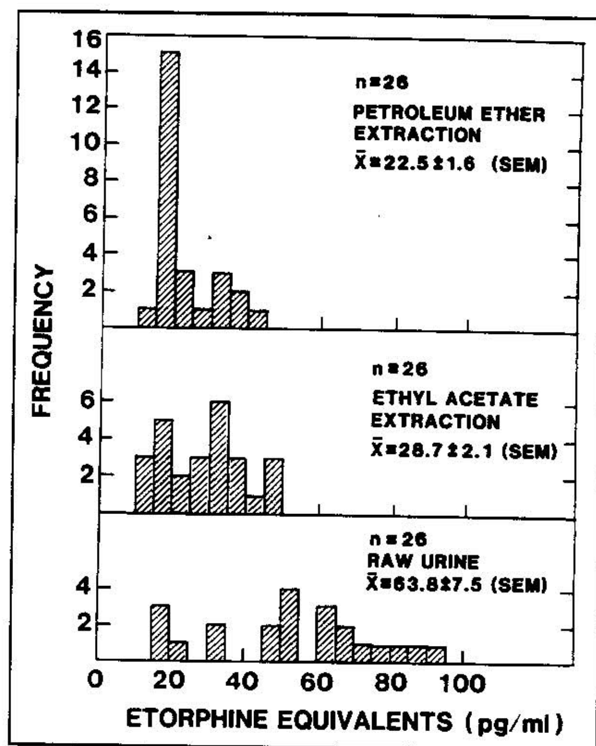

Fig 5—Effect of hydrolysis on background etorphine equivalents. Amounts of apparent etorphine equivalents in 26 postrace urine samples were determined in nonhydrolyzed urine samples (raw urine) and after hydrolysis and extraction into ethyl acetate and petroleum ether.

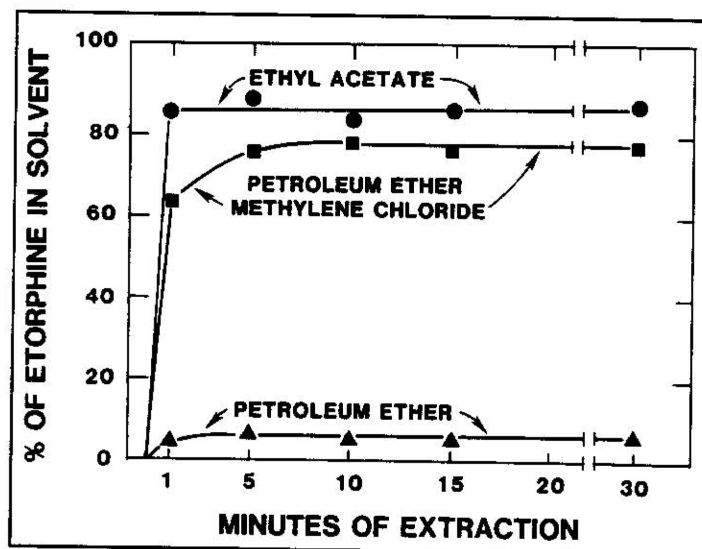

Fig 6—Recovery of ^{3}H-labeled etorphine into ethyl acetate, petroleum ether, and petroleum ether/methylene chloride (2:1).

lyzed urine samples. Hydrolysis reduced the amount of background material in the system by about 50%, but left detectable amounts of etorphine apparently untouched (Fig 8). In this way, hydrolysis increased the sensitivity of the test, and etorphine was detectable in hydrolyzed samples for about 7 days after mares were given 0.22 μg of etorphine/kg.

Interassay precision for high control standards included in our routine assays was 13.3% count variation (n = 15). Intra-assay estimates were not available, because the test was used for routine screening in which

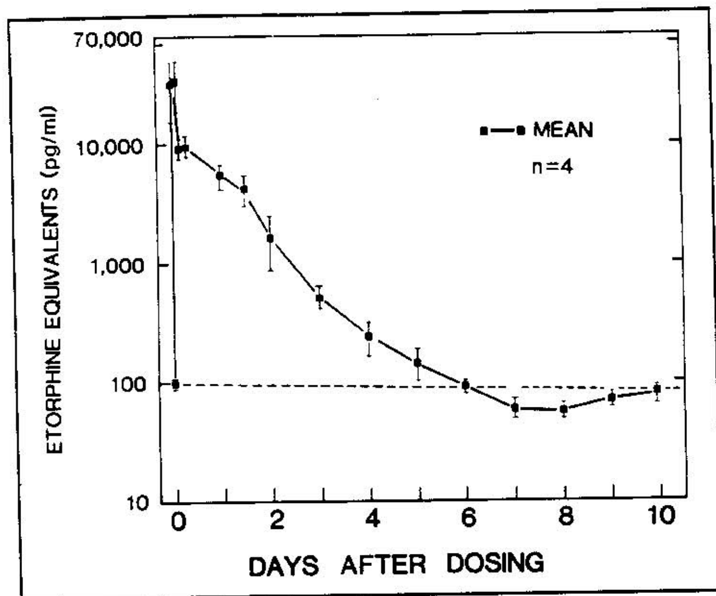

Fig 7—Urinary concentrations of etorphine equivalents after IV administration. Data are expressed as mean urinary concentrations of etorphine equivalents after administration of 0.22 μg of etorphine/kg, IV to 4 mares. Solid lines represent the line of best fit to the mean data points. Dotted lines represent amounts of etorphine equivalents detected in these mares before etorphine administration and after its apparent clearance. Vertical bars represent SEM. Data were obtained in nonhydrolyzed urine samples.

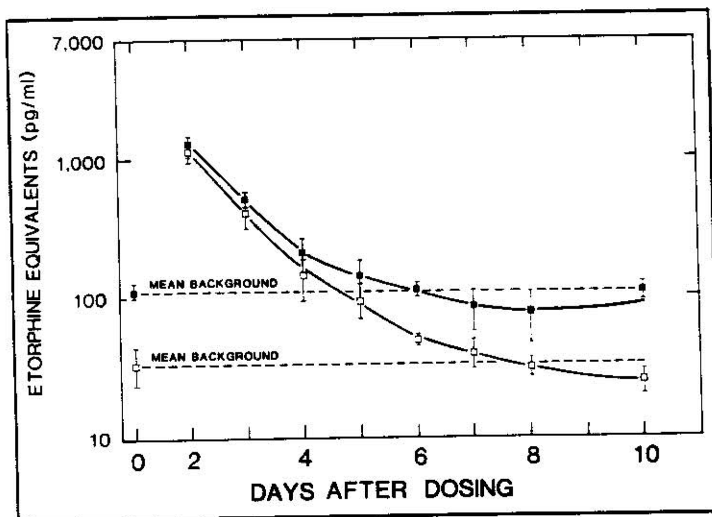

Fig 8—Effect of enzymatic hydrolysis on urinary etorphine equivalents. □—□ = etorphine equivalents (means) observed after hydrolysis of these samples, and ■—■ = nonhydrolyzed samples (mean). Data points represent the mean of the data values obtained at that time. Dotted lines represent etorphine equivalents in these samples before administration of etorphine and after apparent clearance of etorphine.

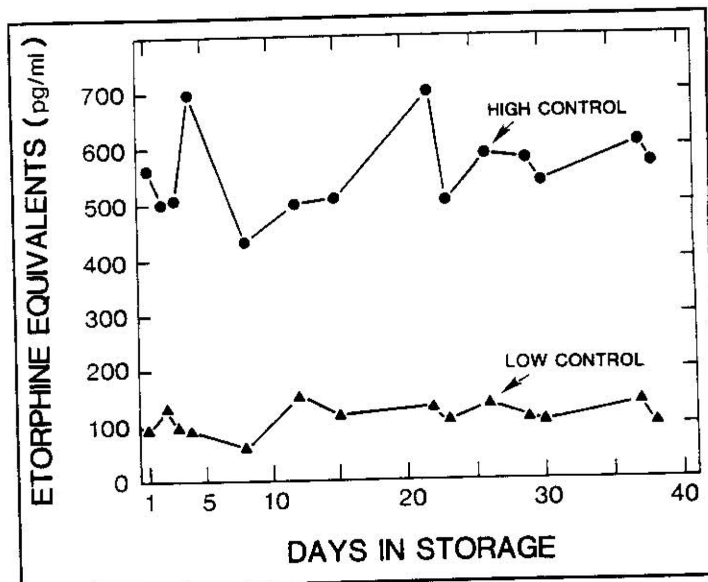

Fig 9—Stability of etorphine equivalents at −20 C. ● and ▲ = amounts of etorphine equivalents in urine samples containing approximately 600 pg/ml (high control) and 100 pg/ml (low control) etorphine equivalents when stored as aliquots at about −20 C for ≤ 38 days.

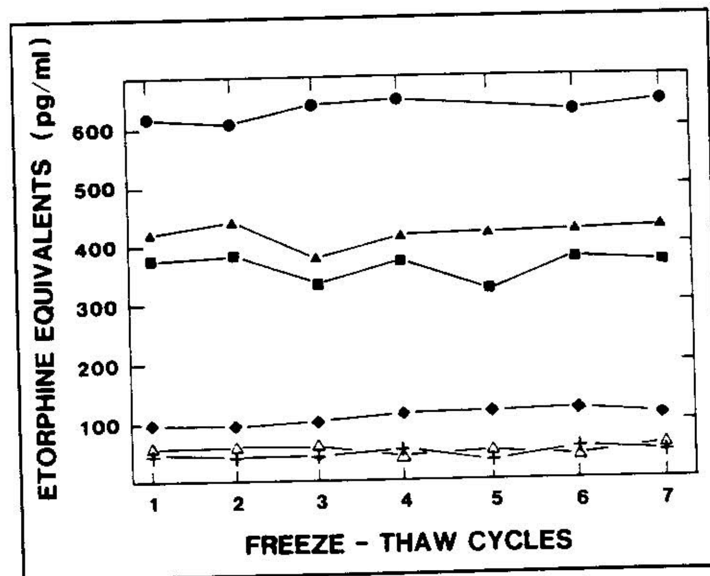

Fig 10—Effect of repeated freezing and thawing on stability of urinary etorphine equivalents. Symbols indicate effects of freezing and thawing on urinary etorphine equivalents in 6 urine samples.

runs are short (< 100 tubes) and samples were uniformly negative.

Stability of etorphine equivalents—Because postrace urine samples containing etorphine are commonly stored frozen and because such samples may be used for referee, reference, or other forensic purposes, knowledge of the stability of etorphine in frozen urine samples is of considerable interest. Aliquots of etorphine containing urine samples were stored at about −20 C, and these samples were analyzed by RIA at various intervals. Analysis of these data indicated that there was no apparent decrease in the concentration of etorphine equivalents in these samples over a 38-day period (Fig 9). Similarly, if these samples were subjected to repeated cycles of freezing and thawing, there was no decrease in the concentration of etorphine equivalents detectable in the samples (Fig 10). Data indicated that the breakdown of etorphine and etorphine metabolites under freezer storage or repeated freeze/thaw cycles did not occur for at least 38 days.

Discussion

Large-scale screening of racehorse urine samples for etorphine is a logistic and technical challenge. Concentrations of etorphine in postrace urine samples may be

low, and the occurrence of medication with etorphine also is low. Equine urine also contains materials that interfere in ^{3}H-RIA to give relatively high background values. Because of these considerations, the speed and economy of the test, as well as its sensitivity, are critical. If the test is not economical and easily performed, then it cannot be used on the large number of samples required for random forensic screening. The test also must be sensitive enough to detect the relatively low amounts of etorphine likely to be found in postrace urine samples. Seemingly, the ^{125}I-RIA represents an improvement on the ^{3}H-RIA currently used for detection of etorphine in postrace urine samples.

A major difference between the ^{125}I-RIA and ^{3}H-RIA is in the separation method. When the ^{125}I-RIA was constructed using the charcoal method of separation, substantial amounts of iodinated material remained associated with the urine sample. Therefore, we precipitated the bound ^{125}I-etorphine-antibody complex with a 2nd antibody and counted the radioactivity of the precipitated complex. In this way, the confounding effect of ^{125}I-labeled etorphine remaining bound in the urine samples was avoided, and the quality of the test was improved. After these modifications, the ^{125}I-RIA detected about 20 pg of etorphine/ml in a standard curve, and cross-reactivity of the antibody with other high potency narcotics was not detected.

In the ^{3}H-RIA, the limiting factor in the efficacy has been the amounts of background material detected by the assay. In our laboratory, this background has had a modal value of about 100 pg of etorphine/ml with occasional values of ≥ 800 pg/ml. These background values can be a problem if the amount of etorphine administered to the horse was low, because amounts of etorphine detected after administration of small doses of drug merge indistinguishably into this background value. Etorphine readings on nonhydrolyzed urine samples, using the ^{3}H-RIA would be difficult to distinguish from background material when the reading in the assay decreased < 1 ng/ml.[4]

Values of background material in the ^{125}I-RIA were markedly lower than those observed in the ^{3}H-RIA. In 101 samples, about 99% of the background values were ≤ 250 pg/ml, less than that observed in the ^{3}H-RIA (Fig 4). Because these assays were performed on raw urine, which is the preferred mode for screening large numbers of urine samples, this factor means that the ^{125}I-RIA was potentially twice as sensitive as the ^{3}H-RIA. This degree of sensitivity may be advantageous for an assay that is predominantly used in routine blind screening for small amounts of illegal medication.

A technique that has been used to increase the sensitivity of the etorphine assay by some workers is to hydrolyze the urine sample before the RIA.[k] The rationale behind this step is that hydrolysis and extraction will allow etorphine to be separated from the background material with which it is associated in an nonhydrolyzed urine sample. Therefore, we hydrolyzed a series of postrace urine samples and extracted them to determine the effect of this step on the background material. To be effective, this step should reduce the amount of background material substantially, but allow virtually complete recovery of free etorphine.

In nonhydrolyzed urine samples, amounts of background material ranged up to about 95 pg/ml (Fig 5). In hydrolyzed and extracted urine samples, amounts of background materials were ≤ 50 pg/ml, and in the case of petroleum ether, the distribution was particularly compact. Hydrolysis of urine samples reduced substantially the amounts of background materials in these samples and improved the efficacy of the RIA.

For hydrolysis to improve the efficacy of the RIA, the recovery procedure must optimize the recovery of etorphine released by the hydrolysis procedure, while at the same time reducing recovery of the interfering background materials. Therefore, we determined the ability of different organic solvents to recover free etorphine. Recovery of etorphine by petroleum ether was low, even after repeated extraction. On the other hand, ethyl acetate readily recovered etorphine from our samples, but amounts of background materials extracted into ethyl acetate can be troublesome. Therefore, a combination of methylene chloride and petroleum ether was used routinely for recovery of etorphine from hydrolyzed samples.

When used on raw or nonhydrolyzed urine, the ^{125}I-RIA allowed detection of etorphine in equine urine for up to 5 days after the last dose. For example, when 0.22 μg of etorphine/kg was administered to 4 mares, increased amounts of etorphine equivalents were detectable in urine samples from these mares for about 5 days. Initially, etorphine equivalents in the urine of these mares were about 35,000 pg/ml, in good agreement with information from our laboratory.[4] Thereafter, urine values of etorphine decreased rapidly in the first few hours after dosing, to ≤ 10,000 pg of etorphine equivalents/ml by 24 hours after dosing. Thereafter, values of etorphine equivalents decreased slowly, with an apparent half-life of about 1 day, to reach a value indistinguishable from background values by about 6 days after dosing.

The mean concentration of etorphine equivalents in these mares was about 150 pg/ml, by 5 days after dosing, of which about 100 pg/ml was background material. Although these samples still contained etorphine at 6 days, etorphine equivalents resulting from etorphine were no longer distinguishable from background values. However, hydrolysis and extraction of the sample served to extend the detection of etorphine equivalents contained in these urine samples for 6 and 7 days after dosing. Hydrolysis acted to reduce the amount of background materials to about 50 pg/ml, and allowed the detection of etorphine equivalents in these samples for another 2 days. Seemingly, etorphine remained detectable as etorphine equivalents in mares for at least 7 days after a 0.22 μg/kg dose, and that after about 7 days, the values in an average mare after treatment with these doses was approximately 10 pg/ml above background values.

Because some racing jurisdictions allow freezing of urine samples, and referee samples are routinely held frozen, it is useful to know the stability of etorphine and its metabolites in frozen urine. Therefore, we froze aliquots of urine from mares containing relatively low amounts of etorphine equivalents and assayed these at various times after freezing. A time-dependent reduction in values of etorphine equivalents in samples from our dosed mares was not observed for up to 38 days after dosing. Seemingly, samples stored frozen were sufficiently stable to allow storage and reanalysis.

Another factor sometimes raised is the stability of drugs during freezing and thawing. When a sample is stored frozen and is thawed to allow aliquots to be taken for analytic work, the freezing and thawing process may result in breakdown of the drug or drug metabolites. Etorphine equivalents in urine samples were not affected by repeated freezing and thawing (Fig 10), supporting the concept that referee samples can be stored frozen, and can be subjected to repeated freezing and thawing without suffering any marked loss of activity.

A RIA for etorphine in urine samples that uses ^{125}I-labeled etorphine and an antibody to etorphine-3-hemisuccinate was developed. This RIA can detect about 20 pg of etorphine/ml in vitro. In urine samples, the test is effective down to about 250 pg/ml in nonhydrolyzed urine, at which amount interfering materials in equine urine begin to limit the ^{125}I-RIA efficacy. If the sample is hydrolyzed before analysis, the sensitivity of the test is improved down to about 100 pg/ml. Using ^{125}I-RIA, etorphine can be detected for about 7 days after administration of 0.22 μg/kg to a mare if the sample is hydrolyzed before analysis. Etorphine and its metabolites appear to be stable in frozen urine and also are not affected by several freezing and thawing cycles. Because the pharmacologic effects of etorphine are over within $\leq$ 12 hours of dosing, ^{125}I-RIA can be used to detect illegal use of etorphine in racehorses.

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