# Pharmacology of Narcotic Analgesics in the Horse: Quantitative Detection of Morphine in Equine Blood and Urine and Logit-Log Transformations of This Data

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## SUMMARY

Morphine was detected in equine biological fluids by a combination of liquid-liquid extraction and column chromatography, followed by derivatization and gas-liquid chromatographic assay, using electron capture detector. Recovery of morphine from the equine biological samples was poor. However, despite an overall recovery of less than 20%, this method had a detection limit of 0.2 ng/ml. Addition of 5,000 U of bovine liver $\beta$-glucuronidase/ml of urine enabled detection of the drug in urine for up to 144 hours after horses were given 0.1 mg of morphine/kg of body weight. Morphine was found for at least 24 hours in serum samples. An adaptation of logit-log transformation of gas-liquid chromatographic data for linearization over 3 log units suggested a simple adaptation to existing semiautomated data handling systems.

The narcotic analgesics are often used in equine medicine to control pain and occasionally for their central stimulant actions. Because of these actions, the use of these drugs in performance horses is usually prohibited. Therefore, sensitive and reliable detection methods are needed for the drug in equine blood and urine.

The detection and quantitation of morphine in equine blood and urine are complicated by several factors. In the 1st place, the doses of morphine given to a horse may be relatively small, in the order of 0.1 mg/kg or smaller. Secondly, morphine is, for reasons which are not clear, unusually difficult to recover from equine blood and urine samples. Thirdly, equine urine contains large amounts of mucus and glucuronide derivatives of other compounds which further interfere with the recovery of morphine or its glucuronide metabolites from horse urine.

The necessity for good quantitative data on blood and urinary concentrations of morphine and its metabolites is more important because of the recent recognition of the role of drug concentration in equine forensic chemistry. Because of this, we initiated a study of the quantitative recovery and detection of morphine from serum and urine. In this report, we present an analytical method which can detect morphine as low as 0.2 ng/ml of equine urine and show that this method can be used to study the disposition of small doses of morphine in the horse.

## Materials and Methods

*Animals*—Mature Thoroughbred and Standardbred mares (450 to 600 kg) were kept at pasture, except on days of an experiment when they were housed in box stalls with hay and water ad libitum. Morphine (0.1 mg/kg) was administered iv into the left jugular vein and all blood samples were drawn from the right side in 20-ml Vacutainer tubes[a] containing no added anticoagulant. Urine samples were collected by bladder catheterization. All samples were stored at −20 C until the analysis was performed.

*Reagents and Equipment*—A Varian 3700 gas chromatograph equipped with a [63]Ni electron capture detector was used for all determinations. The detector was operated at 290 C. Separations were done on a 1.8 m × 2 mm siliconized glass column packed with SP 2250-DB on 100/120 Supelcoport.[b] The column oven was maintained at 235 C, and the injector, at 250 C. The column was treated regularly with 50 μl of Silyl-8,[c] and the columns were repacked about once a month. Prepurified nitrogen, the carrier gas, had a flow rate of 30 ml/min; 2 in-line filters were included for removal of traces of oxygen and water vapor.

All glassware was washed in chromic-sulfuric acid cleaning solution,[d] rinsed thoroughly, and allowed to soak overnight in distilled water before further rinsing in distilled water.

Dichloromethane (DCM) and ethyl acetate ("OmniSolve" purity),[e] isopropanol and methanol (nanograde quality), pentafluoropropionic anhydride (PFPA),[e] and morphine sulfate injectable[f] were obtained from commercial sources.

In the column chromatography cleanup of the samples, SilicAR CC-7 Special[g] was used. The silica gel columns were made by packing a small amount of siliconized glass wool in 6-inch disposable Pasteur pipettes. A slurry of CC-7 Special silica in methanol was added to a mark 6 cm from the small tip of the pipette (approx 250 mg). The methanol was removed by placing the tubes on a vacuum line for a couple of minutes. The columns were allowed to air dry at least overnight.
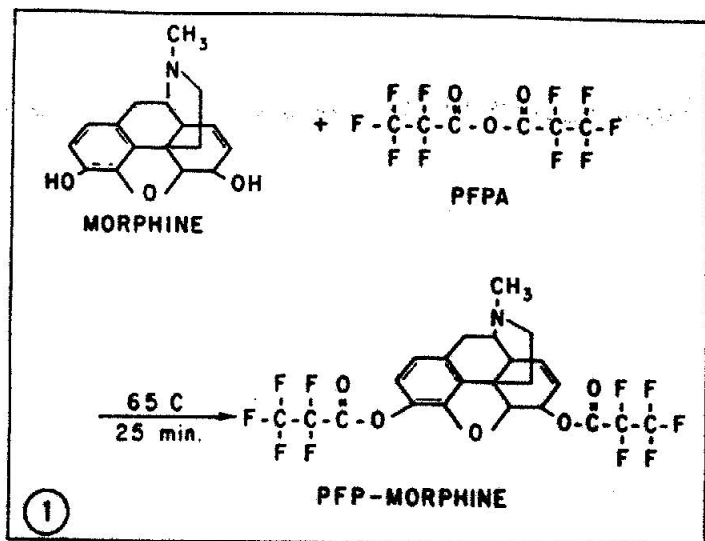
Fig 1—Morphine was converted to a strongly electron capturing compound by a 25-minute incubation at 65 C with 25 µl of PFPA. Mass spectral analysis of the product indicated that the di-derivative of PFP-morphine was formed.



Fig 2—Gas chromatograph of morphine PFPA derivative. The right-hand chromatogram is of a standard solution of 75 ng of derivatized morphine/ml. The left-hand chromatogram is of a urine sample from a horse dosed with morphine and is equivalent to 110 ng of derivatized morphine/ml.

*Urine Sample Analysis*—For samples that were to be hydrolyzed, as much as 2.5 ml of urine was analyzed. The pH was adjusted to 5 with acetic acid and an equal volume of Glucurase[h] was added. (The latter preparation contains 5,000 U of bovine liver β-glucuronidase/ml.) The sample tubes were capped and then incubated for 4 days at 37 C. For nonhydrolyzed samples, as much as 6 ml of urine was used.

The urine samples were then adjusted to pH 8.9 with a 1.5 M sodium carbonate-bicarbonate buffer solution. A DCM-isopropanol mixture (9:1; 4 ml) was added, and the samples were rotoracked for 15 minutes and centrifuged (600 g) for 5 minutes. The aqueous phase was aspirated, and the solvent phase was transferred to a clean centrifuge tube containing 0.5 ml of 0.1N $H_2SO_4$. The samples were again rotoracked for 15 minutes and centrifuged for 5 minutes. The solvent phase was discarded, 1 ml of carbonate buffer was used to bring the solution to pH 8.9, and 4 ml of DCM-isopropanol was added. Back extraction was performed as previously. The DCM-isopropanol phase was separated and allowed to drip through the CC-7 silica gel columns.[f] The columns were washed with five 2-ml aliquots of ethyl acetate-methanol-glacial acetic acid (8:1:1). Excess fluid was removed by forcing air through the column for a few seconds. Morphine was eluted off the columns into clean tubes with 2 ml of ammonia water (2 drops of $NH_4OH$/ml of water). (The pH of the eluate was checked to ascertain that it was approximately 8.9.) The drug was extracted into 4 ml of DCM-isopropanol as before. The solvent was transferred to a 15-ml conical glass tube and evaporated to dryness under a stream of $N_2$.

To derivatize the morphine (Fig 1), 25 µl of PFPA was pipetted into the dried tubes. The tubes were capped and placed in a waterbath (65 C) for 25 minutes. Excess reagent was removed by evaporation under a stream of $N_2$. The residue was redissolved in 25 µl of ethyl acetate by vortexing for 30 s, and a 2-µl aliquot was immediately injected into the gas chromatograph. All values were corrected for percentage of recovery.

*Serum Sample Analysis*—Serum samples (4 to 5 ml) were analyzed in a manner similar to that used for nonhydrolyzed urine samples. When less than 4 ml of serum was needed, following pH adjustment, a single extraction into DCM-isopropanol was performed, and the solvent poured through the silica gel column. Further cleanup was unnecessary for most serum samples.
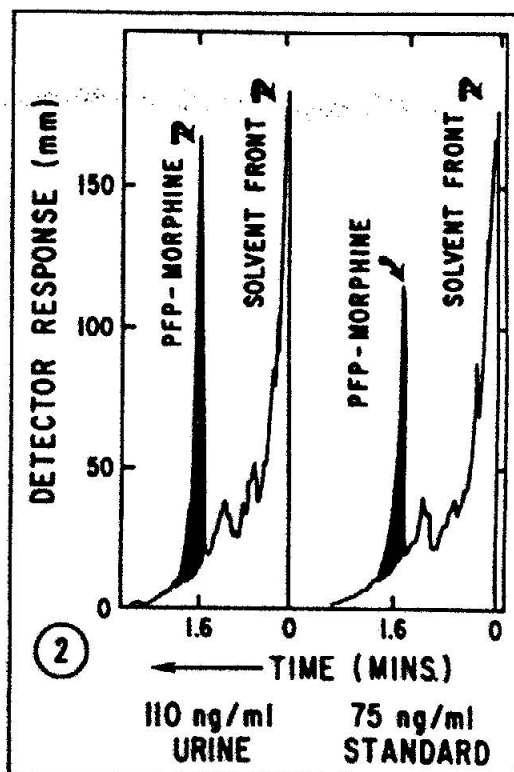
*Studies Using [³H]Morphine*—[³H]Morphine[i] was used to trace sources of loss throughout the extraction procedure. It was also used to study the effects of changing the pH and the extraction times. Final extracts were mixed with 5 to 15 ml of 3a70[j] scintillation cocktail and counted for 5 to 10 minutes in a Beckman LS-3150 T.[k]

*Calculations*—Morphine standard curves were made by an adaptation of the logit transformation of gas-liquid chromatographic (GLC) peak areas. Logit, or logistic probability, was calculated from the equation:

$$\ln [B/(1 - B)]$$

where $B$ = peak area/(2 × peak area of highest standard). Since "2 × the peak area" was used as a maximum and this value affected the slope, for consistency, the 150-ng standard was chosen as the highest standard. Logit values of peak areas were plotted on the $Y$ axis against log values of each standard on the $X$ axis, using logit log paper.[l] The standard curve was calculated as the least squares linear regression line through these points.

## Results

In the bulk of the experimental work reported here, the morphine was reacted with PFPA to obtain a morphine-PFPA derivative (Fig 1), which was then detected by gas chromatography. This morphine-PFPA derivative chromatographed with a retention time of about 1.6 minutes under the conditions described in Materials and Methods (Fig 2). The identity of this material as a di-PFPA derivative of morphine was confirmed by mass spectrometry.

Preliminary experiments showed relatively poor recoveries of morphine added to serum or urine samples. Because of this, studies of optimal pH and extraction times for recovery of morphine from equine serum and urine samples were carried out. Figures 3 and 4 show that the optimal pH from equine samples was about 9.0. Studies on extraction times indicated that extraction times of up to 30 minutes for urine or between 15 and 60 minutes for serum gave equivalent recoveries as shown by 1-way analysis of variance (ANOVA) tests, which yielded F values for urine of 2.838 and for serum of 4.649 ($\alpha = 0.02$). However, if the recovery times were prolonged, the efficacy of recovery from serum increased and that from urine decreased.

Despite the care taken to optimize both pH and recovery times in these experiments, the recovery of morphine in these experiments was usually less than 20% of the amount added to the sample. Thus, a study of the steps in the recovery procedure at which drug loss occurred was done (Fig 5).

The source of loss was failure to extract all of the drug from the aqueous sample. A similarly poor recovery of morphine was observed for extraction with DCM-butanol (9:1). A small percentage of the drug remains in the solvent phase when the drug is being extracted into acid (2.4%) and being adsorbed onto the silica gel column packing (2.3%). Washing the silica gel column with ethyl acetate-methanol-glacial acetic acid reduced the loss in this step to less than 5%, and the loss decreased with each successive wash to 0.7% in the 5th wash. Seven percent of the [³H]morphine was not extracted from the ammonia water eluate into the final DCM-isopropanol. About 0.5% of the drug was lost due to irreversible binding to the silica and siliconized glass wool of the silica gel column. An overall recovery of 18.1% of [³H]morphine was obtained in the final solvent. A GLC determination indicated an overall recovery of 17%.

To ensure optimal derivatization of morphine recovered from biological samples, the characteristics of the derivatization procedure were studied. The PFPA in an amount of 10 µl (17 µg) was apparently insufficient to derivatize 150 ng of morphine (Fig 6, left panel), whereas 25 µl (42 µg) of PFPA gave the maximum amount of derivatization. Although larger amounts of PFPA appeared to give decreasing derivatization, examination of the chromatographs indicated that excess reagent increased background noise to such a level that part of the drug peak was obscured. A 30-minute exposure to a stream of $N_2$ to remove unreacted PFPA was also tried, and the results are shown in the right hand panel of Figure 6. Again, the same pattern occurs with no significant difference (paired data t test, $t = 0.183$, $\alpha = 0.05$) between brief exposure and 30 minutes under $N_2$.

To study the effects of derivatization time on product yield, samples were derivatized for times varying between 2 and 60 minutes, as shown in Figure 7. Two minutes was insufficient for complete derivatization, but there was no significant difference (1-way ANOVA, $F = 1.416$, $\alpha = 0.025$) among 10-, 25-, and 60-minute derivatization times.
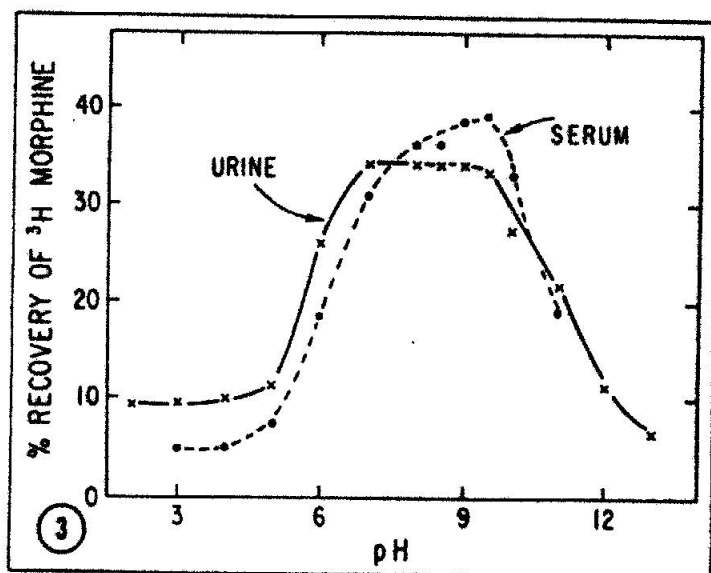


Fig 3—Effect of pH on morphine recovery. Urine and serum samples with added [³H]morphine were analyzed as described in the text. Urine concentrations (×——×) and serum concentrations (●——●) of [³H]morphine at various pH values are shown.
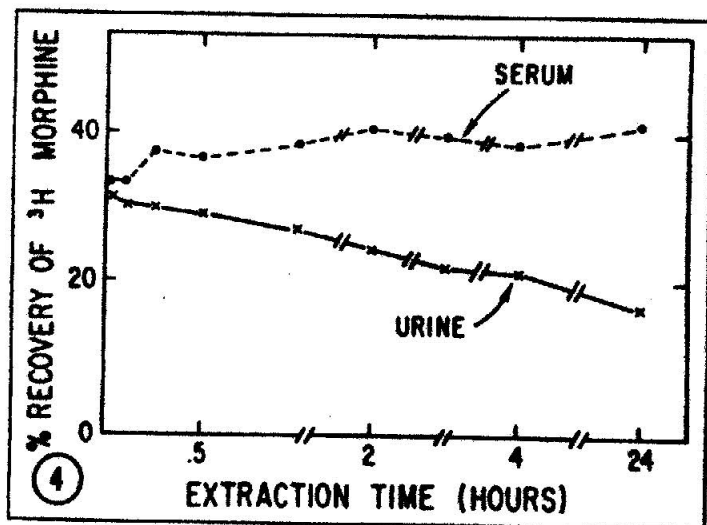


Fig 4—Effect of extraction time on morphine recovery. Urine and serum samples with added [³H]morphine were analyzed as described in the text. The percentages of [³H]morphine recovered from urine (×——×) and serum (●——●) are shown as a function of time.
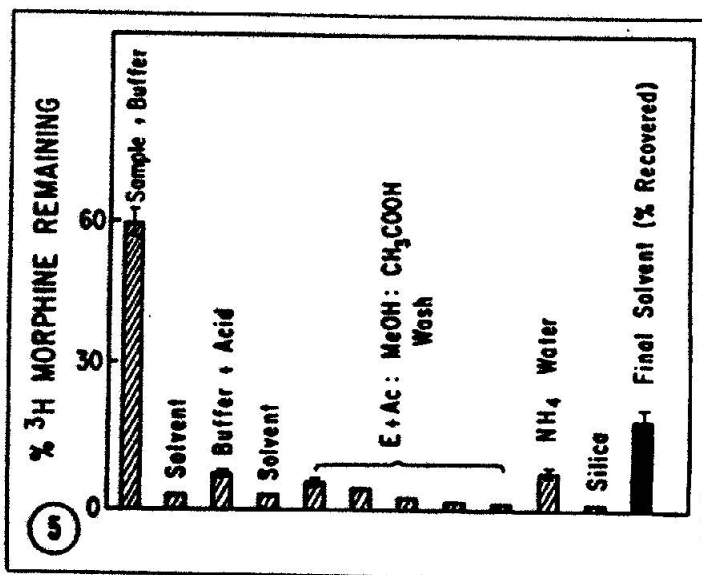


Fig 5—Sources of morphine loss in extraction procedure. Aqueous solutions with added [³H]morphine were subjected to liquid-liquid and column chromatography extractions. The amount of drug lost at each step is presented with SEM.
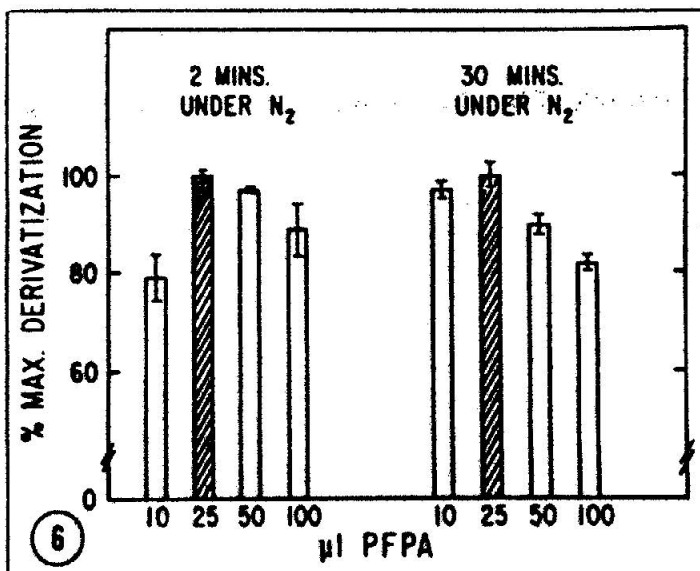
Fig 6—Effect of PFPA concentration on derivative formation. The relative amount of derivative formed following derivatization with various quantities of PFPA is presented. The effect of prolonged exposure to $N_2$ to volatilize excess anhydride was examined. The SEM for each value is included.
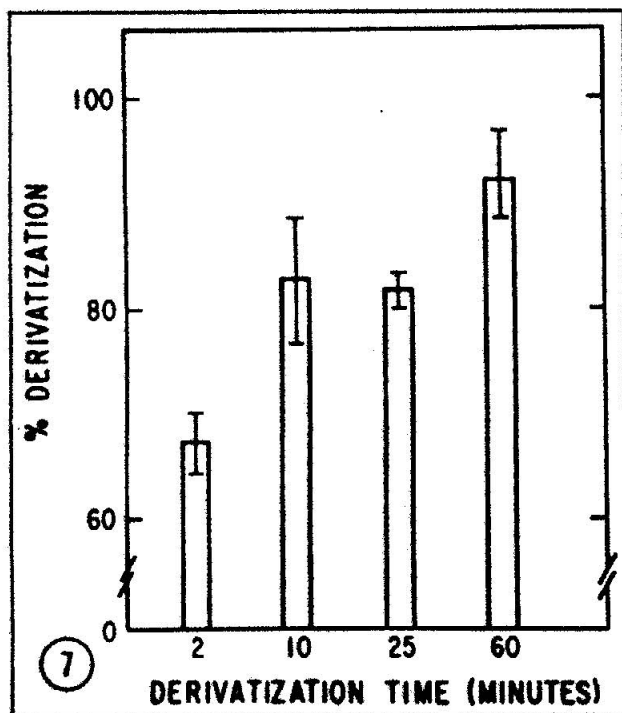


Fig 7—Time course of PFP-morphine derivative formation. The effect of the derivatization time was determined and shows no significant difference (1-way ANOVA, F = 1.416) among 10-, 25-, and 60-minute derivatization times on the amount of PFP-morphine formed (± SEM).

The stability of the pentafluoropropionic (PFP) morphine derivative was checked, and the results are given in Figure 8. A significant loss was noted within 4 hours of derivatization as compared with zero time and almost complete loss by 24 hours to 7.2% of the original value.

Morphine is found in equine urine in 2 forms—as free morphine or as morphine glucuronide. Because the morphine-glucuronide complex is not normally extractable by liquid-liquid extraction, the morphine must first be released from the glucuronide portion of the molecule by acidic or enzymatic hydrolysis. However, for quantitative work, it is important to ensure as complete as possible hydrolysis of the morphine glucuronide; therefore, optimal hydrolytic conditions were determined.

Enzymatic hydrolysis of morphine glucuronide was carried out with 2 variables in mind, the ratio of the volumes of urine to β-glucuronidase and the duration of the hydrolysis period. It was found that a 100:1 ratio of urine to Glucurase resulted in a maximum increase of free morphine of less than 2-fold. In Figure 9, it may be seen that a 10:1
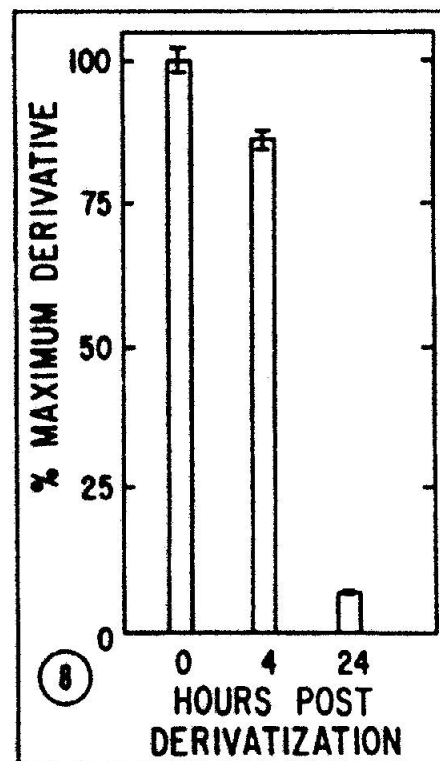


Fig 8—Decay of PFP-morphine derivative with time. Samples were derivatized and allowed to stand at room temperature for various periods before GLC analysis (± SEM).
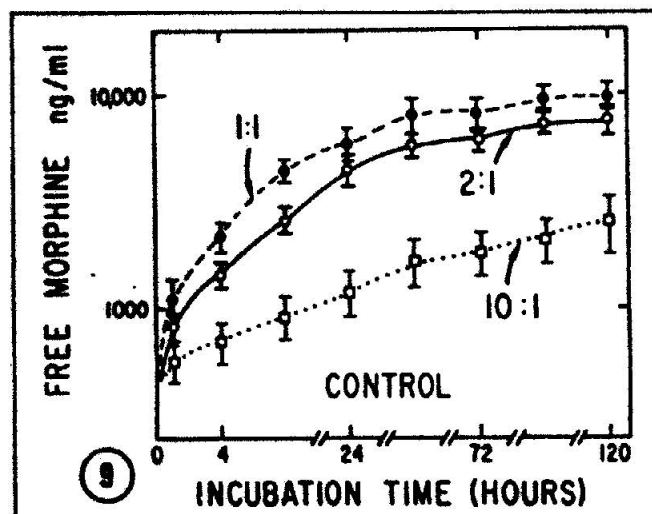


Fig 9—Effect of β-glucuronidase concentration and incubation time on release of free morphine. Urine samples from 4 horses, collected 6 hours after they were given (iv) morphine (0.1 mg/kg), were incubated for various times and with urine to Glucurase ratios of 10:1 (□——□), 2:1 (O——O), and 1:1 (●——●) (± SEM).
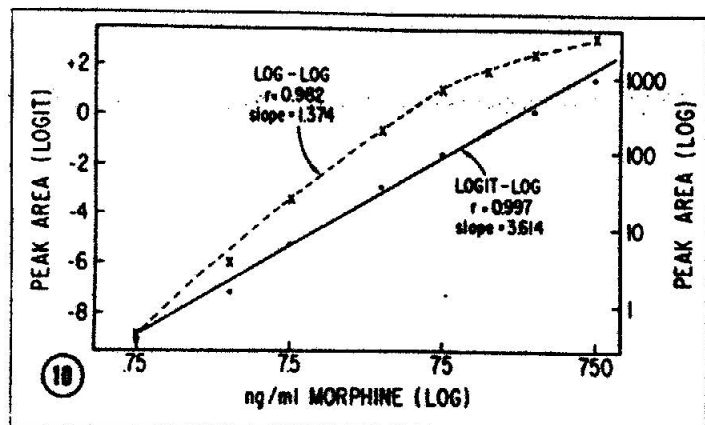
Fig 10—Log-log and logit-log standard curves. Areas under GLC peaks of a series of standards were submitted to both log transformation (×——×) and logit transformation (●——●). Both were plotted against nanograms of morphine/milliliter on a log scale.

until the drug was no longer detected in either hydrolyzed or nonhydrolyzed samples from any horse (at 168 hours). Overall, an average of 12% of the measured morphine was excreted in the free form and 88% was released by β-glucuronidase.

The inset to Figure 11 shows the ratio of the amount of morphine released by β-glucuronidase to the free morphine. This ratio is dependent on the time elapsed from drug administration, increasing steadily to a peak at 9 hours after dosing. Thereafter the ratio decreased. By 120 hours, apparently most of the drug detected in the urine was present in the free form.

The average serum morphine concentrations for the same horses are presented in Figure 12. The values decreased from an average of 301 ng/ml at 3 minutes to 10% of that value at 1 hour and just under 1% of that value at 6 hours. Morphine was detected in the serum of the 4 horses at 24 hours after dosing, but in only 1 horse at 48 hours.
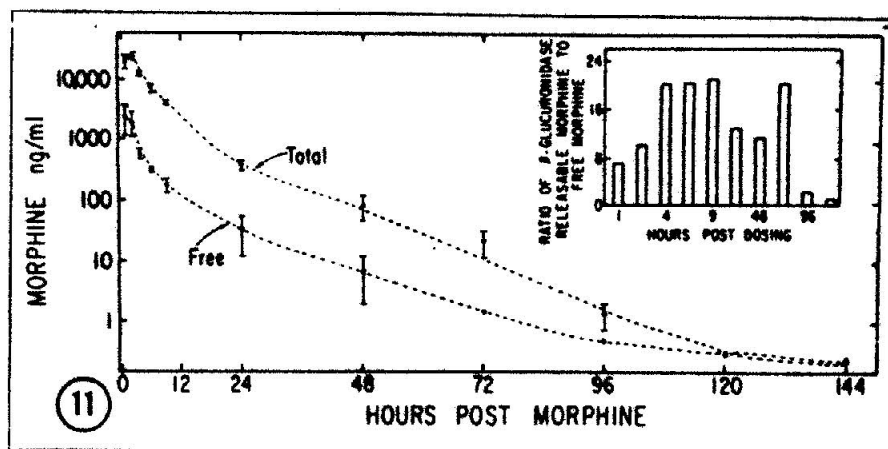


Fig 11—Urine concentrations in horses given (iv) 0.1 mg of morphine/kg. Each sample was analyzed for free morphine following enzymatic hydrolysis (●——●) and without hydrolysis (O——O). The ratio of the β-glucuronidase releasable morphine to free morphine is shown in the inset (± SEM).

ratio produced an average 5.6-fold increase in the amount of free morphine after 120 hours of incubation. The maximum increase in the amount of morphine uncoupled from glucuronide was 21.7-fold following a 120-hour incubation period of urine-Glucurase mixture (1:1). This was a statistically significant increase over a 2:1 ratio (paired data $t$ test, $t = 5.611$, $\alpha = 0.05$). The amount of β-glucuronidase was further increased to 5 parts of the enzyme per 1 part of urine, and an average 34-fold increase in free morphine was found.

A typical standard curve plotted on both logit-log and log-log scales is presented in Figure 10. Conversion of the GLC peak areas to logit units produced a linear relationship between the peak areas and drug concentration over a range of 3 log units. Plotting the same data on a log-log scale resulted in linearity over 2 log units and a slope that was half that of the logit-log standard curve.

Four horses were given (IV) morphine at the dose level of 0.1 mg/kg; blood and urine specimens were obtained at intervals during the next 168 hours. Figure 11 shows the urinary concentrations of free morphine both with and without enzymatic hydrolysis. Urinary excretion peaked at 2 hours after dosing at a mean concentration of 21,894 ng/ml with hydrolysis, and at 1 hour at a mean concentration of 2,367 ng/ml when the samples were not hydrolyzed. Values decreased rapidly during the first 24 hours; thereafter, there was a more gradual decrease in the excretion rate,



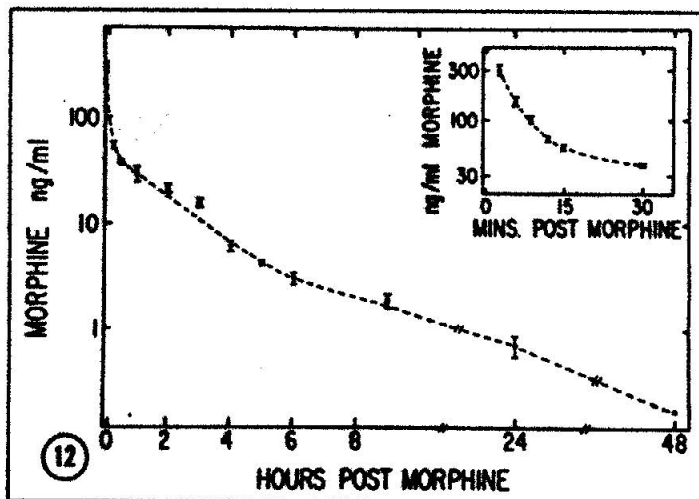Fig 12—Serum concentrations in horses given (iv) 0.1 mg of morphine/kg. The inset shows serum morphine concentrations for the first 30 minutes on an enlarged time scale. (± SEM).

## Discussion

In 1960, Lloyd et al[1] were the first to demonstrate that morphine analysis by GLC was feasible. Since then, colorimetric,[2] spectrophotometric,[3] fluorometric,[4] and enzyme immunoassay and radioimmunoassay[5] techniques have been

used for the quantitative determination of morphine in biological specimens. The GLC is presently the most widely used method because of its improved sensitivity and specificity.[6-13]

Equine biological samples presented special problems. Goblet cells in the epithelium and compound tubular glands in the mucous coat of the equine renal pelvis secrete mucus.[14,15] Consequently, urine from a horse is among the most difficult biological fluids to "cleanup" adequately when attempting very sensitive assay methods. When derivatizing with multifluorinated reagents, the cleanup procedure becomes critical, since many substances present in biological materials possess amino or hydroxyl functional groups which will undergo the same reaction as the drug. A number of extraction techniques were investigated before concluding that a combination of 2 extraction procedures, as outlined in Materials and Methods, was necessary. Although several research workers have reported methods in which the biological sample was applied directly to column chromatography,[16,17] we found that a preliminary liquid-liquid extraction was necessary. Several solvent systems were examined, including chloroform-isopropanol (9:1) and toluene-butanol (9:1). Although some research workers have reported better recovery with chloroform than with DCM,[12] we found that the dirtier background and the increased health risks[18] were unacceptable. The low volatility of toluene-butanol was the deciding factor in the elimination of that solvent. For all solvents tested, the inclusion of a small amount of an alcohol decreased losses of drug due to adsorption onto glassware, as reported by other investigators.[6,7]

Column chromatography packing materials investigated included Florisil, aluminum oxide, charcoal, and the silica CC-7 Special. Florisil gave results similar to those obtained with the silica compound, whereas aluminum oxide resulted in poor recovery of the drug. Although charcoal proved to be a superb cleaning material, it suffered from the problem that the morphine became irreversibly bound under our experimental conditions. The bed length of the column was relatively unimportant. A 15-cm column did result in less recovery, but likely because the volume of eluting fluid was not increased. Washing the column with water-methanol-ethyl acetae (10:3:2)[19] was somewhat less effective than the ethyl acetate-methanol-glacial acetic acid (8:1:1). The volume of washing fluid was not critical, but a larger volume generally produced a cleaner chromatogram. Successive acid washes caused only minor loss of the drug (Fig 5). Elution with methanol alone or in combination with a nonpolar solvent, as suggested by several research workers,[4,17] always resulted in an unacceptably dirty eluent. A single 2-ml aliquot of ammonia water recovered 99.1% of the extractable drug. A 2nd elution recovered an additional 0.7%, and a 3rd elution resulted in the recovery of another 0.2%.

Morphine is in its least charged form at pH 8.9.[7] Therefore, most investigators adjust the pH of the aqueous phase close to this. Figure 3 indicates that some variation in the pH will not result in a significant loss of morphine recovered. The extraction time also was not critical as shown in Figure 4 and accounts for the wide range of extraction times that are reported in the literature: from 3 minutes[12] to 30 minutes.[7] Urine extraction times ranging between 1 and 30 minutes produced no significant change in percentage of recovery, while serum could be extracted in 15 to 60 minutes with no significant difference in recovery percentages.

As illustrated by Figure 5, the greatest loss of morphine is incomplete extraction from the original aqueous phase. Bhargava[20] suggested siliconization of all glassware coming in contact with aqueous solutions of morphine. He reported an increase from 46.5% to 79.4% recovery of morphine upon siliconization. Although we also found greater recovery of drug from siliconized glassware, an increased background level observed on the chromatogram made this an unacceptable alternative. These findings are in agreement with a study done by Blake.[m] Further evidence supporting the fact that the greatest loss is taking place in the preliminary liquid-liquid extraction step was found in other experiments. For example, in one approach, the liquid-liquid extraction steps were omitted; [³H]morphine was added directly to the DCM-isopropanol to be poured through the silica gel column. In this case, 66% of the total counts was recovered at the end of the procedure.

Although previous research workers experimented with on-column derivatization, most recent work uses prechromatographic derivatization.[12] Derivatization procedures using pyridine[13] were not attempted, since pyridine readily contaminates the detector.[12] Direct derivatization with a fluorinated acylating agent of the phenolic and alcoholic hydroxyl groups to form a strongly electron capturing compound is the most widely used approach. Wallace et al[12] reported a detection limit of 25 ng of morphine/ml following the formation of the trifluoracetylated (TFA) derivative, but Felby[6] reported that the electron capture detection is 6 times more sensitive to PFP than TFA derivatives. Heptafluorobutyryl anhydride has been used in the analysis of street samples.[11] However, most researchers have found that the formation of the PFP derivative of morphine is the most satisfactory.[6-8,10] A number of variations in the derivatization method were attempted. The use of 0.2% triethylamine in benzene as a catalyst invariably produced a dirty chromatogram. Furthermore, a catalyst appeared to be unnecessary. There was no observable difference in the degree of derivatization between using 25 $\mu$l of PFPA as opposed to adding 25 $\mu$l of ethyl acetate with the PFPA. More complete derivatization was obtained at 65 C, as opposed to room temperature, and doing the procedure at room temperature did not provide a significantly cleaner chromatogram. The amount of PFPA used was important, as illustrated by the left panel of Figure 6; 10 $\mu$l was insufficient to derivatize 150 ng of morphine. There appeared to be a slight decrease in recovery with 50 or 100 $\mu$l of PFPA, but this was not statistically significant (1-way ANOVA, $F = 2.986$, $\alpha = 0.025$). It is likely that incomplete removal of excess reagents caused an increase in the background, rather than an actual incomplete derivatization. The right panel of Figure 6 indicates that a 30-minute exposure to a stream of $N_2$ did not improve the results. Apparently, interfering compounds were nonvolatile under these conditions. The residue was redissolved in 3 different solvents—ethyl acetate, toluene, and isopropanol. Ethyl acetate gave the best recovery and the OmniSolve grade of this solvent gave a minimum of interfering peaks.

Felby[6] reported that the PFP derivative was stable with no decrease in peak height after storage at room temperature for 4 days. Dahlstrom et al[6] and Dahlstrom and Paalzow[7] reported that the stability of the PFP derivative was good with "only some" loss after storage at 4 C for 3 days.

Submitting the samples to 30 minutes under a stream of N₂, the authors found a significant loss of derivative in only 4 hours (Fig 8) and almost total loss of PFP-morphine within 24 hours. Consequently, samples were derivatized in groups of 4 or less and were chromatographed within 30 minutes. Blau and King[21] suggested that atmospheric moisture will hydrolyze the derivative.

In persons, 8% to 10% of the morphine is excreted free and 90% to 92% is bound as the glucuronide.[22,23] This compares with 12% free and 88% bound of the recovered drug found in the present study. From the data presented in Figure 11, it may be seen that as much as 95% of the detected morphine was released by β-glucuronidase, with the percentage of bound dependent on the time after dosing. Uncoupling of the morphine from its glucuronide can, therefore, markedly increase the concentration of detectable morphine which may be crucial when screening for the low concentrations of morphine which may be administered to a horse. Since acid hydrolysis generally results in unacceptable dirty specimens,[9] morphine is probably best cleaved from its glucuronide by an enzymatic method. In persons, the ratio of urine samples to β-glucuronidase is in the order of less than 1:1.[24] Figure 9 clearly demonstrates that a far lower ratio is required when dealing with equine urine. It has been suggested that other glucuronides in horse urine compete for the available β-glucuronidase catalytic sites.[25] Although 5 parts of β-glucuronidase to 1 part of urine was found to increase free morphine levels 34-fold, this much reagent becomes prohibitively expensive when screening large numbers of samples on a daily basis.

A possible pretreatment of the urine to enable the use of smaller quantities of the Glucurase has been suggested.[9,26] Sulfate ions that may interfere with the enzyme action can be precipitated by the addition of 10% BaCl₂ (w/v). Furthermore, the specific β-glucuronidase inhibitor, saccharo-1,4-lactone, is removed by bringing the urine to pH 11.5. Frey et al[9] found that after this pretreatment, 2 ml of urine plus 0.1 ml of β-glucuronidase incubated overnight resulted in essentially complete uncoupling of the morphine-glucuronide complex in human urine. Attempts to repeat this using equine urine failed, no difference being observed between pretreated and untreated urine. Perhaps the β-glucuronidase inhibitors in equine urine are different or this system may be overwhelmed by a large number of competing glucuronides.

Although the inset of Figure 11 indicates that the percentage of morphine excreted as the glucuronide is highest at 9 hours after dosing, one untested factor must be considered, at least for a minor role. Perhaps there are naturally occurring components of equine urine which can hydrolyze bound drug. When there is a sufficiently small amount of drug present, all of the drug is hydrolyzed, such as in the 5th- and 6th-day samples. A few hours after dosing, when a large amount of drug is present, the activity of this component may be swamped and any natural hydrolysis is undetectable.

Although most workers used a 3% OV-17[5-8,11,12] or 3% OV-22[10] column, we found the relatively new SP 2250-DB gave shorter retention times and a better chromatogram in terms of peak shape. Like other workers,[6,7,11] we found that regular treatment of the column with Silyl 8 helped to prolong the life of the column. The SP 2250-DB required a higher column oven temperature than that generally used for the analysis of PFP-derivative morphine on an OV-17 column.

Typical standard curves plotted arithmetically or on a semilog scale are linear over less than 1 log unit. Interpolation of a nonlinear standard curve is difficult and inaccurate. Log-log treatment of the data does give linear results over almost 2 log units, which may be adequate in some situations, but particularly for pharmacokinetic work, a wider range is often desirable.

Logit-log conversion of data to obtain straight line standard curves has been common practice for radioimmunoassay work since Rodbard et al[27] first introduced the idea over 10 years ago. The wide acceptance of this method of data analysis may be attributed to the relative ease with which the data may be entered into an automated data handling system. To the authors' knowledge, logistic probability has not been applied to GLC data. The authors have used this calculation method in a study of phenylbutazone concentrations and procured excellent correlation with values obtained on the same samples by an independent laboratory using conventional calculation methods. Confirmation of the validity of these interconversions was obtained as follows. Standard curves were plotted for 15 to 150 ng on logit-log, log-log, semilog, and arithmetic graphs. Logit-log, log-log, and arithmetic values for a list of data points were calculated from the least squares linear regression line. The semilog plot was not sufficiently linear, so values were read off the graph manually. Values obtained by these 4 methods were tested in a 1-way ANOVA and the correlation among them was excellent, with F equal to 0.005. In place of logit, the probit transform could also be used.

Since many workers using gas chromatography have access to existing automated data systems, a modification of the logit-log program would be inexpensive and eliminate the time-consuming calculations often associated with quantitative GLC.

# References

1. Lloyd HA, Fales HM, Highet PF, et al: Separation of alkaloids by gas chromatography. *J Am Chem Soc* 82:3791, 1960.
2. Fujimoto JM, Way EL, Hine CH: A rapid method for the estimation of morphine. *J Lab Clin Med* 44:627–635, 1954.
3. Mule SJ: Determination of narcotic analgesics in human biological materials. *Anal Chem* 36:1907–1914, 1964.
4. Kupferberg H, Burkhalter A, Way EL: A sensitive fluorometric assay for morphine in plasma and brain. *J Pharmacol Exp Ther* 145:247–251, 1964.
5. Spector S: Quantitative determination of morphine in serum by radioimmunoassay. *J Pharmacol Exp Ther* 178:253–258, 1971.
6. Dahlstrom B, Paalzow L, Edlund PO: Simultaneous determination of codeine and morphine in biological samples by gas chromatography with electron capture detection. *Acta Pharmacol Toxicol* 41:273–279, 1977.
7. Dahlstrom B, Paalzow L: Quantitative determination of morphine in biological samples by gas-liquid chromatography and electron-capture detection. *J Pharm Pharmacol* 27:172–176, 1975.
8. Felby S: Morphine: Its quantitative determination in nanogram amounts in small samples of whole blood by electron-capture gas chromatography. *Forensic Sci Int* 13:145–150, 1979.
9. Frey DE, Wills PD, Twycross RG: The quantitative determination of morphine in urine by gas-liquid chromatography and variations in excretion. *Clin Chim Acta* 51:183–190, 1974.
10. Kogan MJ, Chedekel MA: Rapid g.l.c. method for the separation of picogram quantities of morphine and codeine. *J Pharm Pharmacol* 28:261, 1976.
11. Moore JM: Rapid and sensitive gas chromatographic quantitation of morphine, codeine, and O⁶-acetylmorphine in illicit heroin using an electron capture detector. *J Chromatogr* 147:327–336, 1978.
12. Wallace JE, Hamilton HE, Blum K, et al: Determination of morphine in biologic fluids by electron capture gas-liquid chromatography. *Anal Chem* 46:2107–2111, 1974.
13. Yeh SY, Gorodetzky CW, Krebs HA: Isolation and identification of morphine 3- and 6-glucuronides, morphine 3,6-diglucuronide, morphine

3-ethereal sulfate, normorphine, and normorphine 6-glucuronide as morphine metabolites in humans. *J Pharm Sci* 66:1288–1293, 1977.

14. Catcott EJ, Smithcors JF (ed): *Equine Medicine and Surgery*, ed 2. Wheaton, Ill, American Veterinary Publications Inc, 1972, p 377.

15. Sisson S: *The Anatomy of Domestic Animals*, ed 4. Philadelphia, WB Saunders Co, 1953, p 569.

16. Breiter J, Helger R, Lang H: Evaluation of column extraction: A new procedure for the analysis of drugs in body fluids. *Forensic Sci Int* 7:131–140, 1976.

17. Sprague GL, Takemori AE: Improved method for morphine extraction from biological samples. *J Pharm Sci* 68:660–662, 1979.

18. Casarett LJ, Doull J (ed): *Toxicology: The Basic Science of Poisons*. New York, Macmillan Publishing Co Inc, 1975.

19. Stolman A, Stewart CP: Isolation and determination of morphine, codeine and heroin from viscera and body fluids by adsorption. *Analyst* 74:536–542, 1949.

20. Bhargava H: Improved recovery of morphine from biological tissues using siliconized glassware. *J Pharm Sci* 66:1044–1045, 1977.

21. Blau K, King GS (ed): *Handbook of Derivatives for Chromatography*. London, Heyden & Son Ltd, 1978, p 126.

22. Paerregaard P: Excretion of morphine in the urine of non-addicts. *Acta Pharmacol Toxicol* 14:53–57, 1957.

23. Payte JT, Wallace JE, Blum K: Hydrolysis: A requisite for morphine detection in urine. *Curr Ther Res* 13:412–416, 1971.

24. Fish F, Hayes TS: Hydrolysis of morphine glucuronide. *J Forensic Sci* 19:676–683, 1974.

25. Tobin T, Miller JR: The pharmacology of narcotic analgesics in the horse. I. The detection, pharmacokinetics and urinary "clearance time" of pentazocine. *J Equine Med Surg* 3:191–198, 1979.

26. Shackleton CHL, Charro-Salgado AL, Mitchell FL: Urinary neutral steroid profile analysis in adults and infants. *Clin Chim Acta* 21:105–118, 1968.

27. Rodbard D, Bridson W, Rayford PL: Rapid calculation of radioimmunoassay results. *J Lab Clin Med* 74:770–781, 1969.