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Furosemide, Patella vulgata β-Glucuronidase and Drug Analysis: Conditions for Enhancement of the TLC Detection of Apomorphine, Butorphanol, Hydromorphone, Nalbuphine, Oxymorphone and Pentazocine in Equine Urine.

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ABSTRACT

We have investigated the action of five sources of β-glucuronidase enzymes on the hydrolysis of glucuronides of apomorphine, butorphanol, hydromorphone, nalbuphine, oxymorphone and pentazocine in equine urine.

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For all glucuronides tested, Patella vulgata β-glucuronidase yielded the largest thin layer chromatographic (TLC) spots. For oxymorphone, P. vulgata was the only treatment to yield detectable TLC spots under test parameters.

For these six drugs, TLC spot size and chromatographic quality were compared between control horses and horses pretreated with furosemide four hours earlier. Furosemide pretreatment produced a statistically significant increase in spot size and was found to enhance chromatogram quality.

These findings support previous suggestions that P. vulgata is a superior drug-glucuronide hydrolyzing enzyme. They also support earlier reports that administration of furosemide at four hours prerace is unlikely to result in significant interference with routine drug testing procedures.

INTRODUCTION

Furosemide (Lasix®) is the treatment of choice for exercise-induced pulmonary hemorrhage (EIPH) or epistaxis (Tobin, 1981). The only problem with this approach is that furosemide may interfere with the detection of certain drugs in urine for a period after its administration.

In 1977, Gabel, Tobin, Ray and Maylin (1977) suggested that this interference or dilution effect would be minimal four hours after furosemide administration. Recently, Combie and co-workers (1981) confirmed

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this estimate, showing that after 0.4 to 0.5 mg/kg furosemide IV the dilution effects were over in three hours or less. The present study was undertaken to extend the scope of this earlier work. Thin layer chromatography was chosen as the testing mode because this is the most widely used drug screening method. The drugs examined were apomorphine, butorphanol, hydromorphone, nalbuphine, oxymorphone and pentazocine.

Current screening methodology used by the Kentucky Equine Drug Detection Laboratory includes hydrolysis of urine samples by β-glucuronidase from P. vulgata before analysis by thin layer chromatography. Although previous work has shown that the P. vulgata is the preferred source of β-glucuronidase for cleaving morphine glucuronide (Combie, Blake, Nugent, Tobin, 1981), no studies have yet reported the applicability of this procedure to cleave other drug-glucuronide complexes. Therefore, samples were hydrolyzed by β-glucuronidase from five different sources, including P. vulgata, to determine whether the Patella preparation remained superior in the presence of other aglycones.

MATERIALS AND METHODS

Apomorphine was obtained from Eli Lilly & Co, Indianapolis, IN; butorphanol was from Bristol Laboratories, Syracuse, NY; hydromorphone was from Wyeth Laboratories, Inc, Philadelphia, PA; nalbuphine and oxymorphone were from Endo Laboratories, New York, NY; pentazocine was from Winthrop Laboratories, New York, NY; and furosemide was from National Laboratories Corp, Somerville, NJ.

Experiment 1:

Six mares were dosed intravenously (IV) with one of the following preparations: 0.012 mg apomorphine per kg, 0.05 mg butorphanol

(Stadol®) per kg, 0.01 mg hydromorphone per kg, 0.14 mg nalbuphine (Nubain®) per kg, 0.005 mg oxymorphone (Numorphan®) per kg, and 0.25 mg pentazocine (Talwin-V®) per kg. A urine sample was obtained 75 min later by bladder catheterization from the horse dosed with oxymorphone. Urine samples were obtained from the other five horses 50 min after dosing.

Five sources of β-glucuronidase from Sigma Chemical Co, St. Louis, MO, were utilized: P. vulgata (Type L-II), Helix aspersa (Type HA-4), Helix pomatia (Type H-1), bovine liver (Type B-3) and Glucurase®. The first four were lyophilized powders stored at -20 °C which were weighed out and dissolved in distilled, deionized water to make solutions equivalent to 5000 U of β-glucuronidase per mL immediately before an assay was run. Glucurase® was a bovine liver β-glucuronidase solution, acetate buffered to pH 5 and stored at 4 °C. It was used as obtained from the manufacturer. Urine samples to be hydrolyzed by either H. aspersa or H. pomatia were adjusted to pH 4.5; those to be hydrolyzed by P. vulgata or bovine liver were brought to pH 5; and those to be incubated with the Glucurase® were adjusted to pH 5.5 with acetic acid.

Hydrolysis was performed by mixing 200 µL of urine from all horses, except the one given oxymorphone, with 200 µL of each enzyme solution and incubating for 1 h in 1 mL sealed, glass ampules in a water bath. For oxymorphone, 400 µL of urine and an equal volume of enzyme were incubated. An incubation temperature of 65 °C was used for samples containing P. vulgata as the β-glucuronidase source, while all other samples were incubated at 55 °C.

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Following incubation, 200 µL of the urine-β-glucuronidase mixture (600 µL for samples containing oxymorphone) was mixed with 500 µL of a 1.5 M carbonate buffer, bringing the pH to 8.9 with the exception of urine from the horse dosed with apomorphine. Urine containing apomorphine was adjusted to pH 7. The buffered urine samples were extracted with 4 mL dichloromethane:isopropanol (9:1). Following separation by centrifugation, the solvent was transferred to clean tubes and 1 mL of 0.1 N H_2SO_4 was added to each. The tubes were mixed gently for 15 min. The layers were separated by centrifugation and the solvent was discarded. Two mL of the carbonate buffer was added to each tube, bringing the pH to 8.9 except for apomorphine-containing samples. For samples from horses dosed with apomorphine, only 0.5 mL of the buffer was added to adjust the pH to 7. Four mL of fresh solvent was added to each tube. Following extraction and separation by centrifugation, the dichloromethane: isopropanol was transferred to a clean set of tubes and evaporated to dryness under a stream of nitrogen.

The residue was redissolved in 25 µL of dichloromethane and spotted on high performance thin layer chromatography plates (Brinkmann Instruments, Inc, Westbury, NY; E. Merck, manufacturer) pre-coated with silica gel 60, using long capillaries drawn from 22.9 cm Pasteur pipettes. The plates were developed in ethyl acetate:methanol:glacial acetic acid (80:10:10). The solvent front was allowed to traverse a distance of 5 cm. Following air drying, the plates were sprayed with modified Folin-Denis reagent.[1]

[1] A mixture of 10 g sodium tungstate, 2 g 12-molybdosilicic acid, 5 mL concentrated phosphoric acid and 50 mL water was refluxed for 2 h. The mixture was diluted to 100 mL with additional water and stored at 0-5 °C (can be stored at room temperature).

The plates were then exposed to fumes of ammonium hydroxide to maximize the blue-green color reaction.

The plates were labeled with a code unknown to the person reading them. A score of 10 was assigned to the largest, most distinct spot for each drug. Failure to detect the presence of a drug was recorded as a zero. The other spots were then ranked on this 0 to 10 scale by the independent reader.

Experiment 2:

TABLE I

Experimental Design

-4 h	administered saline (control) or 0.4 mg furosemide/kg; water withheld.
-45 min	administered nalbuphine, oxymorphone, pentazocine.
-30 min	administered butorphanol, hydromorphone.
-20 min	administered apomorphine.
0 min	POST TIME (hypothetical).
+20 min	allowed water ad libitum.
+30 min	urine sample collected.
+ 1 h	urine sample collected.
+ 2 h	urine sample collected.

Six mares were dosed with saline for the control part of this experiment and on a separate occasion were given 0.4 mg furosemide (Lasix®) per kg for the test section of this experiment. This was done in random order. The time of administration of the saline or the diuretic was set at -4 h, corresponding to 4 h before hypothetical race time. Water was withheld

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at this time. Each horse was given one of six drugs in the same dosages as listed in Experiment 1. The timing of this drug administration ranged from 20 to 45 min before post-time as listed in Table I. Twenty min after post time, waterbuckets were returned to the stalls housing these horses and they were allowed water ad libitum for the duration of the experiment. Urine samples were collected by bladder catheterization at 30 min, 1 h and 2 h after the time of the hypothetical race. Samples were stored at -20 ° C until they could be assayed. The specific gravity of all samples was determined.

Urine samples were adjusted to pH 5. Either 80 µL or 500 µL of urine was incubated with an equal volume of a 5000 U/mL solution of β-glucuronidase from P. vulgata for 3 h at 65 °C. The hydrolyzed urine samples were assayed as outlined under Experiment 1. The samples were assigned a number with the aid of a random number generator. The plates were read and scored by one of us (JMB) with no knowledge of either the code or the experimental design. A score of 10 was given for the largest, most distinct spots for the entire experiment. Failure to detect the presence of a drug was assigned a zero and other spots were ranked on this 0 to 10 scale.

RESULTS

The ability of β-glucuronidase from 5 sources to hydrolyze six different drug-glucuronide complexes was checked (Fig 1). For all six drugs, hydrolysis by the P. vulgata preparation, gave the maximum size spot on the TLC plate and was assigned a score of 10. With the exception of apomorphine, use of β-glucuronidase from the powdered bovine liver resulted in the lowest score, indicating that for 5 of the drugs in this study, the

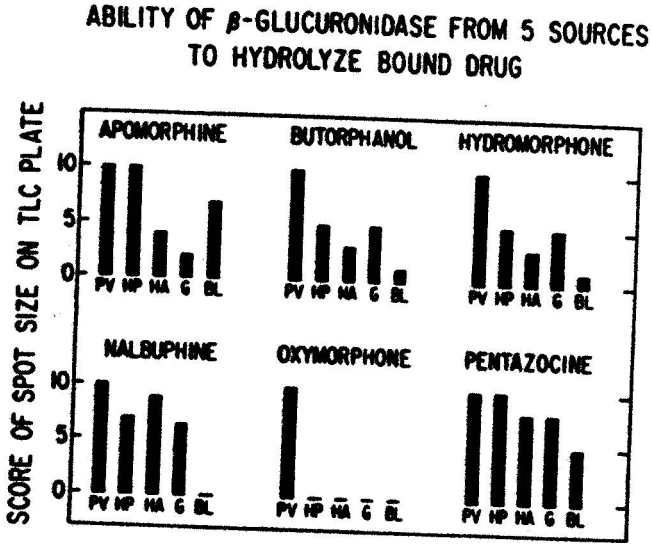

Fig 1 The ability of β-glucuronidase from 5 sources (PV - Patella vulgata; HP - Helix pomatia; HA - Helix aspersa; G - Glucurase®; BL - powdered bovine liver) to hydrolyze six different drug-glucuronide complexes is shown as the score of spot sizes on TLC plates following a 1 h hydrolysis.

bovine liver preparation had the poorest ability to hydrolyze the drug-glucuronide complex. β-glucuronidase from H. pomatia, H. aspersa and the Glucurase® solution, gave intermediate results with the exact order of the scores of TLC spot sizes varying somewhat among the six different drugs. The choice of the source of β-glucuronidase appeared to be most critical for oxymorphone, where the drug was detected only in the sample submitted to hydrolysis by the P. vulgata enzyme under parameters of experiment 1. All other enzyme preparations resulted in insufficient hydrolysis for the presence of oxymorphone to be detected. The amount of pentazocine administered to the horse was sufficient to give acceptable results regardless of the source of β-glucuronidase.

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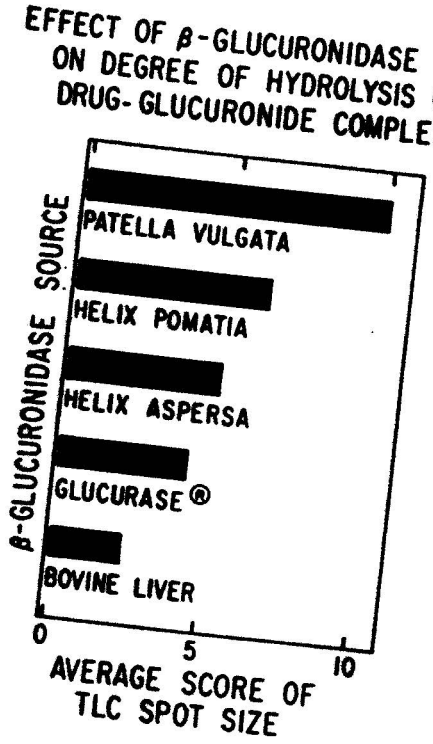

Fig 2 Six mares were dosed IV with one of the following drugs: apomorphine, butorphanol, hydromorphone, nalbuphine, oxymorphone, and pentazocine. Urine samples were hydrolyzed with 5 β-glucuronidase preparations, extracted and spotted on TLC plates. The plates, labelled with a code unknown to the person reading them, were scored on a 0 to 10 scale (10 - maximum size spot, 0 - no drug detected). The average score of TLC spot size is normalized to the scores obtained from the use of Patella vulgata β-glucuronidase.

The mean scores obtained with each individual drug and enzyme preparation are illustrated in Fig. 2. While hydrolysis by the P. vulgata preparation freed the maximum amount of drug in each case, the bovine liver β-glucuronidase hydrolyzed only 23% as much drug-glucuronide complex on the average, resulting in a mean score of 2.3. Of the five β-glucuronidase preparations tested, enzyme from P. vulgata in each case gave the best results, regardless of the aglycone.

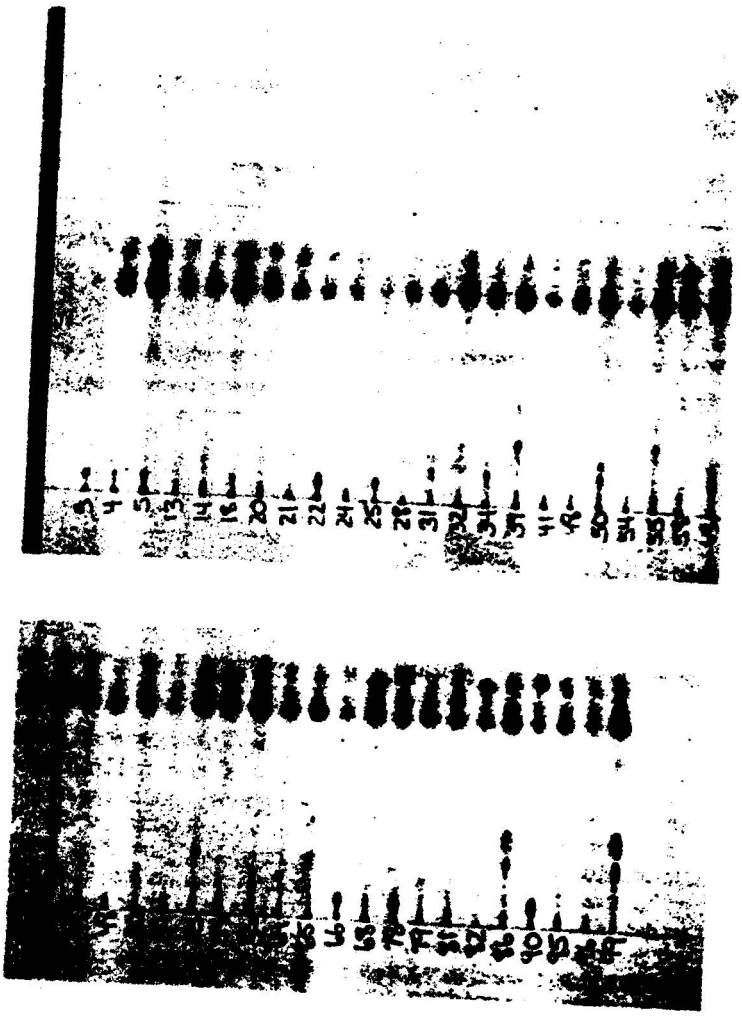

Fig 3 Six horses were pre-treated with saline for the controls and, on a separate occasion, with 0.4 mg furosemide per kg. They were dosed with one of 6 drugs, apomorphine, butorphanol, hydromorphone, nalbuphine, oxymorphone or pentazocine and urine samples were collected according to the schedule in Table I. Samples were hydrolyzed by β-glucuronidase from Patella vulgata for 3 h at 65 °C, extracted and spotted on TLC plates such as the one shown here. Samples were assigned numbers by random number generator to eliminate reader bias.

The effect of furosemide on drug detection is shown in Figures 3 and 4. Figure 3 is a photograph of the TLC plate obtained following analysis of the 500 µL of urine from horses treated and not treated with furosemide before being dosed with one of the six drugs used in this study (Experiment 2). The photo shows the random numbers assigned to the samples. This was one of the plates scored to determine the effect of Lasix® pre-treatment on the size of the drug spot on a TLC plate. Before the code was broken, the test reader noted that some samples appeared to be much "cleaner" than others, and these observations were recorded. Upon analysis of the data, it was found that the samples from horses pre-treated with the diuretic gave cleaner, more distinct and easier to read thin layer chromatograms. For example, although #18 and #58 samples were from the same horse, at the same time following drug administration, and assigned comparable scores, #18 gave a more distinct spot and exhibited the presence of less extraneous material, thus less smearing. Sample #18 was obtained after the horse had been given furosemide, while sample #58 was from the same horse not pre-treated with the diuretic.

The effect of furosemide on the TLC spot size for six different drugs is shown in Fig 4. The values plotted were an average of those obtained following analysis of both 80 and 500 µL of urine. A 1-tailed, paired data t-test indicated that, in this experimental design, administration of furosemide resulted in a statistically significantly higher TLC score ($t = 1.902$, $\alpha = .05$) than control samples. Statistical analysis of the specific gravity of all urine samples in this experiment revealed that the overall slight increase in urine specific gravity following pre-treatment with furosemide was not significant (1-tailed, paired data t-test, $t = .670$, $\alpha = .05$).

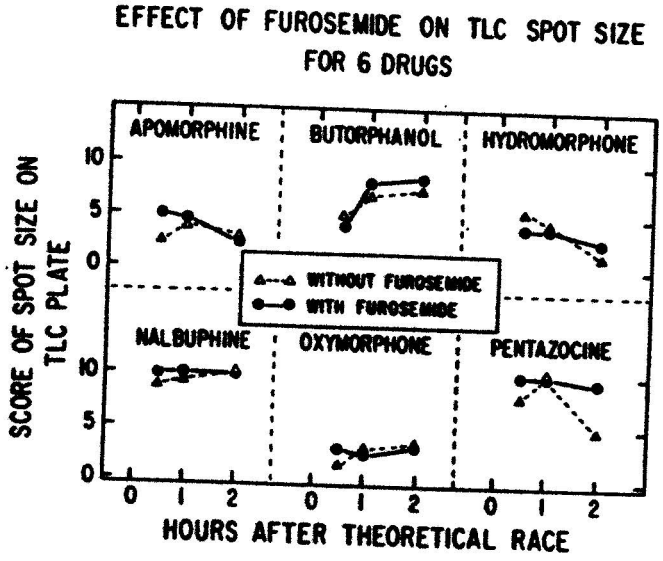

Fig 4 Six mares were pre-treated with saline (shown by the open triangles, Δ-Δ) and, on a separate occasion, with 0.4 mg furosemide per kg (shown by the closed circles, ●-●). They were dosed with one of the six drugs indicated according to the schedule in Table I. Urine samples were hydrolyzed by β-glucuronidase from Patella vulgata. They were analyzed by thin layer chromatography and the spots were scored according to size.

DISCUSSION

P. vulgata was the most useful of the five β-glucuronidase preparations evaluated for activity against the glucuronide metabolites of six different drugs. When 5,000 U of P. vulgata β-glucuronidase was incubated with the drug-glucuronide complexes of apomorphine, butorphanol, hydromorphone, nalbuphine, oxymorphone and pentazocine in 500 μL or less of urine, we found larger TLC spots after use of the P. vulgata enzyme. This held for all drugs tested; one drug, oxymorphone, was detected only after hydrolysis of the urine sample with the Patella vulgata β-glucuronidase under test parameters.

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Based on this experience, we tested the efficacy of this glucuronide hydrolysis system in a situation where the efficient hydrolysis of drug-glucuronide metabolites may be forensically important. The circumstance chosen was pre-treatment of horses with furosemide. This is because the diuresis due to furosemide can dilute out certain drugs and drug-glucuronide metabolites in urine, rendering their efficient recovery important (Tobin, 1981).

In the test model, we elected to treat our horses with furosemide at 0.4 mg/kg four hours "pre-race". Four hours was selected because this is the current Kentucky rule and has been suggested by the Veterinary Chemists' Advisory Committee to the National Association of State Racing Commissioners (Gabel et al, 1977). The test drugs were administered between 45 and 20 min prior to the hypothetical post time. The times of administration of the drugs were selected so that the drugs would have maximal behavioral effects at post time, and the doses selected were below threshold doses for measurable behavioral effects.

One objective of this experiment was to determine whether or not furosemide treatment would interfere with routine post-race TLC screening for drugs in horse urine. For this reason, the extraction and TLC methodology used was similar to that currently used in the Kentucky Equine Drug Testing Program (Blake et al, 1979). All TLC plates were coded and read "blind" by one of us (JWB) to obviate observer bias in the interpretation of these tests.

In these experiments, pre-treatment of horses with furosemide did not appear to significantly reduce the detectability of any of the

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drugs tested. In fact, when the spot size scores were compiled and compared with the control values, the score from the furosemide-treated horses was actually statistically significantly better than in the untreated samples. Further, the reader of the plates commented that the quality of the plates from the furosemide-treated horses was superior to those from the untreated horses. It appears that Lasix® tends to reduce the concentration of materials which would tend to co-extract with the drug, thus leaving a "cleaner" sample. This results in less smearing and better spot definition. The experiment is in good agreement with earlier work which showed that the drug diluting effects of these small doses of furosemide is over within three hours or less of dosing (Combie, Nugent, Tobin, 1981). The experiment also suggests that the earlier recommendation of Gabel, Tobin, Ray and Maylin (1977) on lack of significant drug dilution effect of furosemide at four hours is essentially correct.

Since the diuretic effects of furosemide are characteristically brief, the lack of interference by these doses of furosemide with drug testing was not unexpected. However, the apparently clear-cut enhancement of the quality of the thin layer chromatograms in the presence of furosemide was surprising. On the other hand, previous work from this laboratory has shown that urinary concentrations of fentanyl and its metabolites and free morphine in horse urine were found to be higher four hours after furosemide treatment than in controls (Combie, Nugent, Tobin, 1981), which supports these thin layer chromatographic findings. In association with the fact that some drugs are not affected by the

diluting effects of furosemide, these results show that under some circumstances furosemide may actually enhance the detection of drugs in horse urine.

In summary, therefore, these results support suggestions that P. vulgata β-glucuronidase is a superior drug-glucuronide hydrolyzing enzyme for the structures tested. They further support earlier studies and estimates of the duration of the drug "diluting" effects of furosemide and support suggestions that four hours pre-race is a relatively conservative time to administer this drug.

REFERENCES

1. Blake, J.W., Tobin, T., and Roberts, B.L. (1979). A modified Folin-Denis overspray for the detection of phenolic alkaloidal drugs. Second Intn'l Symposium on Equine Medication control.

2. Combie, J., Blake, J.W., Nugent, T.E., and Tobin, T. (1981). morphine-glucuronide hydrolysis: superiority of Patella vulgata β-glucuronidase as a hydrolyzing enzyme. Clin Chem, in press.

3. Combie, J., Nugent, T.E., and Tobin, T. (1981). The pharmacology of furosemide in the horse. IV. The duration of reduction of urinary concentration of drugs. J Equine Vet Sci, submitted for publication.

4. Gabel, A.A., Tobin, T., Ray, R.S. and Maylin, G. (1977). Furosemide in Horses: A Review. J Equine Med & Surg, 1:215-218.

5. Tobin, T. (1981). "Drugs and the Performance Horse", Charles C. Thomas, Publisher, Springfield, IL, pp 112-115.