Plase

INTRODUCTION

Many drugs used in illegal medication of racing horses are detected in equine urine primarily as their glucuronide metabolites (Miller et al., 1974). Because glucuronide metabolites are poorly lipophilic, they are concentrated by renal concentrating mechanisms. Urinary concentrations of these metabolites may be many times greater than plasma levels of the parent compounds. Because sensitive analytical methods are not available for these metabolites, this urinary concentrating mechanism is important in that it enables analysts to identify many of these drugs during routine forensic screening (Miller et al., 1976).
The diuretic drug furosemide is widely used in racing horses for control of epistaxis (bleeding) (Roberts et al. 1976). Since diuretic drugs markedly interfere with both renal concentrating mechanisms (Roberts et al., 1976) and the active transport of glucuronide metabolites into renal tubules, we decided to investigate the actions of furosemide on urinary concentrations of drugs excreted as glucuronides. Pentazocine was selected for these preliminary experiments as it is rapidly and essentially completely metabolized to glucuronide metabolites in the horse. The results presented here show that furosemide can produce up to a 50-fold decrease in urinary concentrations of glucuronide metabolites of pentazocine. Furosemide treatment, therefore, likely produces marked reductions in urinary concentrations of other glucuronide drug metabolites and may markedly interfere with routine forensic screening for these drugs.

MATERIALS AND METHODS

Mature Thoroughbred horses of between 375 and 550 kg body weight were used. These animals were kept on pasture and stabled in individual loose boxes on the days of the experiments. Prior to and during the experiment, hay and water ad lib were available.

All urine samples were obtained by bladder catheterization. Prior to each experiment, bladders were drained and then emptied completely at the end of each collection period. In this way, both the volume of urine formed and the quantity of drug excreted were estimated for each period. Horses were administered pentazocine at 0.5 mg/kg as rapid intravenous (IV) injection and plasma and urine samples taken. In the test animals pentazocine was followed at 30 minutes by furosemide 1 mg/kg IV, and plasma

*Blake, J. W. Unpublished observations
and urine samples again taken. Urine samples were generally held overnight at 2° C or frozen at -32° if held for longer periods.

Hydrolysis of pentazocine glucuronides was performed by adding to 0.5 ml of urine 1.0 ml of saturated \( \text{NH}_4\text{PO}_4 \) to bring the pH to about 5.0 and 0.8 ml of bovine liver \( \beta \)glucuronidase (Glucerase). These were then incubated overnight at 37° (about 16 hours). Hydrolysis with this relatively large amount of enzyme for this time was required for complete release of all \( \beta \)glucuronidase releasable pentazocine.

At the end of the incubation period, 3-4 drops of concentrated \( \text{NH}_4\text{OH} \) were added to bring the pH of the system to about 9.0. Then 4 ml of dichloromethane was added, the whole rotoracked for 5 minutes, and centrifuged at 1000 x g for 5 minutes. The dichloromethane phase was then separated and evaporated at 55° in a water bath to dryness, 0.5 ml of benzene added and an aliquot of this sample taken for gas chromatography.

Chromatography was on a Perkin-Elmer 3920A equipped with a three-foot OV101 column and a nitrogen phosphorous detector. Injection temperature was 210°, column temperature 220° and manifold temperature 250°. Gas flows were 1 ml/min \( \text{H}_2 \) at 8 psi, 100 ml min/air at 30 psi. The pentazocine peak recovered from equine urine had the same retention time as authentic penta-zocine over three different column temperatures. Derivatization of the material recovered from equine urine with pentafluoropropionic acid (PFPA) also produced material which chromatographed with the similar retention times as PFPA-treated pentazocine.

Furosemide was the injectable form and was a gift of Hoechst-Roussel Pharmaceuticals, Inc., Somerville, New Jersey. Pentazocine was used as
injectable Talwin\textsuperscript{R} and was a gift of the Winthrop Laboratories Division of Sterling Drug, Inc., New York. Liver \(\beta\)-glucuronidase (Glucurase) was from the Sigma Chemical Company, St. Louis. All experimental points are means of four experimental determinations and all lines were fitted by eye.

RESULTS

The upper panel in Fig. 1 shows the diuretic response obtained after administration of 1 mg/kg furosemide. As shown previously (Roberts et al, 1976), this dose of furosemide produced a marked diuretic response, urinary output increasing about 20-fold compared with the control period. The diuretic effect was transient, peaking within the first 30 minutes and declining rapidly thereafter.

The lower panel in Fig. 1 shows urinary concentrations of pentazocine glucuronides after administration of 0.33 mg/kg IV. In the absence of furosemide urinary levels of the drug declined rapidly over the first two hours, then more slowly. Other experiments (data not presented) have shown that the subsequent decline in urinary concentration of pentazocine is very slow, glucuronide metabolites being detectable in equine urine for up to four days post-administration of the drug.

In the presence of furosemide, however, urinary concentrations of the drug drop extremely rapidly, from about 100 \(\mu\)g/ml pre-furosemide to less than 2 \(\mu\)g/ml at 15 minutes post-furosemide. Thereafter, mean drug concentrations in equine urine remained more or less constant at about 1.5 \(\mu\)g/ml for up to one hour, although in some individuals urinary concentrations dropped to about one-hundredth of control levels. Thereafter, urinary pentazocine concentrations returned toward control levels, though they were still less than half control levels at 3.5 hours post-dosing with furosemide.
Fig. 1  Effect of furosemide on urine volume and glucuronide metabolite concentrations.

The upper panel shows urinary output in four horses treated with 0.33 mg/kg pentazocine at indicated zero time (open bars). The solid bars show urinary output in four horses treated with the same dose of pentazocine at zero time and 1 mg/kg of furosemide IV at thirty minutes. Bars represent the total volume of urine collected in the preceding time period, i.e. thirty minutes or one hour. The lower panel shows urinary concentrations of glucuronide metabolites at each time point in control horses (open circles, O-O) and those treated with 1 mg/kg furosemide (solid squares, ■■■). All data points are means ± S.E.M. of experiments on at least four horses.
DISCUSSION

At a dosage level of 1 mg/kg IV, furosemide produced up to a 20-fold increase in urine volume in four Thoroughbred mares. These changes in urinary output were associated with a 50-fold drop in urinary concentrations of glucuronide metabolites of pentazocine. Because of the spread of urinary concentrations in furosemide-treated animals, concentrations of pentazocine in some urines were reduced to about one-hundredth of levels observed in controls.

These observations are consistent with theoretical expectations. Because glucuronide metabolites are highly water soluble and of marginal lipid solubility, they are ideal candidates for concentration by renal concentrating mechanisms. Indeed, this concentrating mechanism is vital for the forensic detection of apomorphine in equine samples, as the routine detection of concentrations of this drug found in plasma is very difficult (Miller et al., 1976). The forensic importance of this concentrating mechanism prompted these experiments and the observed dilution confirms some prior suggestions (Tobin, 1977).

As well as affecting renal concentrating mechanisms, other mechanisms exist by which furosemide may act to reduce urinary concentrations of glucuronide metabolites. Because glucuronide drug metabolites contain acidic carboxyl groups, they are likely to be pumped into equine urine by the organic acid transport mechanism (Hirsch, 1976). Furosemide, by occupying sites on this transport mechanism, is likely to reduce this transport of glucuronides and thus further reduce their concentration in equine urine. The very large reductions (up to 100-fold) in urinary concentration of glucuronide observed
in some of the experiments reported here suggest that this mechanism may be an important factor, but further experiments are required to clearly demonstrate such an interaction.

In the experiments reported here, it was relatively easy to detect the diluted concentrations of pentazocine metabolites in furosemide-treated urines, and it might therefore be argued that this dilution effect is not forensically significant. It should be borne in mind, however, that these experiments were performed under optimized conditions with sensitive detection methods. The dose of pentazocine given was relatively large, and furosemide was administered only 30 minutes later. The conditions under which the urine samples were hydrolyzed with $\beta$-glucuronidase were carefully optimized, and complete release of all pentazocine available for release by $\beta$-glucuronidase was obtained. Finally, use of a nitrogen detector enabled relatively specific detection of the pentazocine molecule with much less interference from other substances than was observed using electron capture detection. These considerations contributed to a clear-cut demonstration of the dilution effect with considerable reserve detection capacity at maximal dilution. Under routine forensic conditions, however, circumstances are likely to be considerably less than optimal and a 50-fold diluting effect would become highly significant. Further, it is likely that the diluting effect applies to other drugs, such as apomorphine, the phenothiazines, fentanyl and other narcotic drugs, with which there is likely to be much less margin for dilution. In good agreement with this probability are some recent experiments by Ozog*, who reports substantial reductions in concentrations of urinary apomorphine in

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*Ozog, F. W. Personal communication, 1977. Dept. of Chemistry, St. Regis College, Denver, CO.
furosemide-treated horses, supporting the hypothesis that glucuronide metabolites are particularly susceptible to diuretic dilution (Tobin et al, 1977).

Recently, Frey and co-workers (1976) reported on the use of bumetanide in obtaining urinary samples for dope testing in horses. These workers concluded that the use of bumetanide "did not interfere with the detection of doping drugs" and that "by enhancing the clearance of drugs used for doping, bumetanide even provides favorable conditions for the detection of such drugs". The results reported here do not appear to support these conclusions. Since furosemide and bumetanide are both acidic, high ceiling diuretics which act in the same way and by the same mechanism to produce diuresis, it appears highly likely that both will have similar effects on urinary concentrations of glucuronide drug metabolites. Further, bumetanide reduces urinary concentrations of phenobarbital (Frey et al, 1976) and furosemide produces substantial reductions in urinary concentrations of phenylbutazone. These observations suggest that the conclusions of Frey et al (1976) should be treated with caution. Because sample size is limited in the routine dope testing situation, changes in the urinary concentrations of the magnitude reported in this and previous reports (Roberts et al, 1976) are critical for routine drug testing and may mean the difference between success and failure.

As a practical matter, these results show that administration of furosemide to pentazocine pretreated horses resulted in a 50-fold reduction in urinary concentrations of glucuronide metabolites of this drug. The effect was transient, peaking at about one hour and drug levels were approaching control levels by about the fourth hour. These reductions in concentration are
more than sufficient to interfere with the detection of pentazocine under routine screening conditions. Of further importance, these results make it likely that urinary concentrations of many other drugs excreted as glucuronide metabolites, such as apomorphine, narcotics and the phenothiazine tranquilizers, will also be affected by treatment with furosemide and other diuretics. The results suggest that furosemide may be used to dilute out drugs other than the previously reported phenylbutazone and suggest that racing authorities should strictly control its use. The results also suggest further limitations on the usefulness of furosemide to produce diuresis in horses which are reluctant to urinate in the test barn following a race.
REFERENCES


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