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Short Communication

Effect of Urine pH on Urine Levels of Oxyphenbutazone in Racing Horses

The use of phenylbutazone in racing horses is often regulated by quantitating urine levels of phenylbutazone and its metabolites. Oxyphenbutazone is one of the major metabolites of phenylbutazone found in equine urine (1). Because of the forensic importance of the levels of oxyphenbutazone found in equine urine, we elected to investigate the effects of urinary pH on blood and urine levels of oxyphenbutazone in racing horses.

Urine pH in racing horses is unusual in that it may range from pH 4.5 to pH 10.0. Beyond having such a wide range, the distribution appears to be bimodal, with population peaks at about pH 5.0 and pH 8.0 (1). This wide range of urine pH values in racing horses suggests that urinary levels of either phenylbutazone or its metabolites may be substantially influenced by urinary pH (2) independently of others factors such as the time which has elapsed since the last dose of phenylbutazone. Despite the well recognized role of pH in affecting the distribution and disposition of drugs (1, 2), and its potential forensic importance (1), only two studies in this area have been reported (3, 4).

To determine the effect of pH on urine concentrations of oxyphenbutazone, we measured blood and urinary levels of oxyphenbutazone in post-race samples from thoroughbred horses racing in Kentucky. The data show that while oxyphenbutazone levels in equine blood are not significantly influenced by urine pH, urine concentrations of oxyphenbutazone are highly dependent on urine pH and may vary up to 500-fold, depending largely on whether the urine is acidic or basic.

Materials and Methods

Phenylbutazone and oxyphenbutazone were obtained from Ciba Pharmaceuticals (Summit, NJ) and γ-hydroxyphenylbutazone were obtained from Ciba-Geigy (Switzerland). Liquid chromatographic grade methanol and water were from Burdick-Jackson (Alltech, Deerfield, IL). All other solvents and reagents used were of analytical grade from Fisher Scientific (Louisville, KY).

All blood and urine samples tested were post-race blood and urine samples submitted to the Kentucky Equine Drug Testing Laboratory by the Kentucky State Racing Commission. Blood samples were drawn and urine samples collected when the horse voided urine post-race. All samples were stored at 4°C and shipped to the University of Kentucky for analysis within 48 hr. Phenylbutazone is a permitted medication for thoroughbred horses racing in Kentucky.

A Beckman 341 liquid chromatograph equipped with a 421 controller, Altex model 100 pump, and an Altex model 153 UV detector (254 nm) was used. A 20-μl loop was used on the 210 sample injector valve. An Ultrasphere-ODS 5-μ column (4.6 mm × 25 cm) with a guard column (Pellicular C-18, 4.6 mm × 5 cm, Alltech) between the injector and analytical column was used for the separation of the compounds.

The pH of the urine was measured on a Fisher Accumet Model 230 pH meter when the samples were received in the laboratory.

The chromatographic procedure used was based on that described by Marunaka *et al.* (5). The mobile phase was a linear gradient of 50% methanol–50% 0.01 M sodium acetate (pH 4) as the initial concentration and 100% methanol (at 5%/min) as the final concentration. The column was maintained at room temperature with a flow rate of 1.0 ml/min and the eluted compounds were recorded by the detector at 254 nm.

Blood samples were collected in 20-ml Vacutainer tubes containing potassium oxalate and sodium fluoride. Urine samples were collected in glass jars when the horses voided. To 1 ml of plasma was added 4 ml saturated KH_2PO_4 and 6 ml dichloromethane at room temperature. The samples were processed by Rotorack for 6 min and centrifuge for 2 min at 2000 rpm and the organic layer was transferred to a clean tube and evaporated to near dryness in a water bath (65°C). The samples were completely dried under a stream of N_2. To the residue, 100 μl methanol was added and 20 μl of the sample injected onto the column. The urine samples were prepared in a similar manner, except 4 ml of pH 3.3 saturated KH_2PO_4 was added.

Standard curves were prepared for the determination of phenylbutazone, oxyphenbutazone, and γ-hydroxyphenylbutazone by adding known amounts of authentic standards to blank plasma or urine and assaying by the same extraction procedure. Concentration ranges of 0.5–5.0 μg/ml and 10–50 μg/ml were used. Peak heights were plotted against the concentration.

Results

The insert in fig. 1*A* shows the distribution of urine pH values observed in the 110 post-race urine samples in which oxyphenbutazone was quantitated. The pH values observed ranged from about 4.8 to 8.5. The distribution observed is consistent with the distribution of pH values in several thousand post-race horse urines reported by Moss *et al.* and Makajima *et al.* (1). The pH range represented in fig. 1*A* covers the great bulk of the range reported by these authors.

Fig. 1*A* shows the matched plasma and urinary levels of oxyphenbutazone found in these horses, plotted against urinary pH values. The data show that the plasma levels of oxyphenbutazone do not vary with pH since similar plasma levels were found in the urine of horses forming acidic or basic urines. The data suggest that the plasma levels of oxyphenbutazone in the horse are not substantially influenced by urine pH.

In contrast, urine concentrations of oxyphenbutazone were very variable and highly dependent on urinary pH. At a urinary pH 6.0 or less, plasma and urinary levels of oxyphenbutazone were broadly similar. On the other hand, at pH 8.0 and above, urinary concentrations of oxyphenbutazone averaged 50 μg/ml or more, about 50 times the plasma levels of oxyphenbutazone observed in these horses.

When these data were replotted such that the ratio of the urine to plasma levels of oxyphenbutazone were presented against pH, these ratios increased logarithmically with increasing pH value (fig. 1*B*). These data suggest that the urinary concentrations of oxyphenbutazone in racing horses are strongly influenced by urinary pH.

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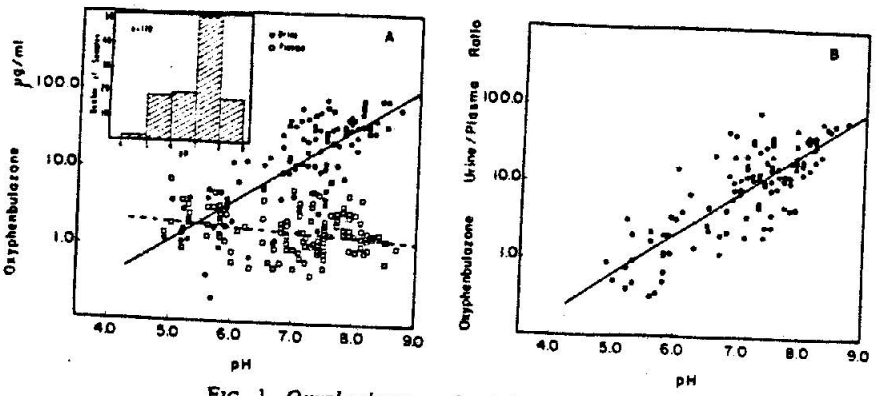

FIG. 1. *Oxyphenbutazone levels in racing horses.*

A, urinary pH and plasma and urine levels of oxyphenbutazone. The *solid circles* and *lines* show urine levels of oxyphenbutazone plotted against urinary pH ($r = 0.77$), while the *open squares* and *dashed line* show concentrations of oxyphenbutazone in the corresponding plasma samples ($r = -0.16$). The *inset* shows the frequency distribution of pH values of the urine samples used in these experiments. *B*, urine/plasma ratio of oxyphenbutazone. The data from *A* has been plotted as the ratio of urine/plasma oxyphenbutazone concentrations at the given pH ($r = 0.80$). All lines were fitted by the method of least squares.

Discussion

These experiments suggest that the concentrations of oxyphenbutazone in post-race urine samples of thoroughbred horses racing in Kentucky were highly influenced by urinary pH. If the pH of the urine sample was below pH 6.0, the blood and urinary concentrations of oxyphenbutazone were comparable. On the other hand, if the pH of the sample was 8.0 or above, the urinary concentrations of oxyphenbutazone approached 100 μg/ml, while the plasma levels of oxyphenbutazone remained low. The data suggest that oxyphenbutazone levels in equine urine may vary up to 500-fold, depending principally on the pH of the urine sample.

These data appear to represent a classic example of the "ion-trapping" phenomenon. As an acidic drug, the concentrations of oxyphenbutazone are high in basic urines and low in acidic urines. Using a transformation of the Henderson-Hasselbalch equation, the pK_a of oxyphenbutazone (4.7) and the known 4.5 to 10.0 range of pH values observed in equine urines, one can readily calculate an expected range of oxyphenbutazone concentration in equine urines. Based on these calculations (1, 2), one may expect about a 10,000-fold range in oxyphenbutazone concentration in equine urines, given a single plasma level of free oxyphenbutazone.

Because the proportion of bound to free oxyphenbutazone in equine plasma is not known, it is not possible to calculate the actual plasma to urine ratio of oxyphenbutazone which would be found in these horses.

Beyond being a classic demonstration of the ion trapping phenomenon, these data are forensically important. This is because phenylbutazone use in racing horses is often regulated by measuring urinary concentrations of phenylbutazone and its metabolites. Despite wide academic acceptance of the effects of urinary pH on urine concentrations of drugs in horses, the potential effect of urinary pH on this regulatory process was not recognized forensically. These data, however, demonstrate a 500-fold variation in urinary levels of oxyphenbutazone depending on urinary pH. It appears likely that given more sensitive analytical instrumentation and the wider range of pH values obtained in larger numbers of samples, a somewhat greater range of oxyphenbutazone levels in equine urine would be observed.

Since all these samples were racing samples, the doses of phenylbutazone or other drugs which may have been administered to these horses are not known. However, the plasma levels of oxyphenbutazone observed in these experiments are relatively low and do not vary significantly with pH. On the other hand, urinary levels of oxyphenbutazone varied directly with pH and in a manner qualitatively consistent with that predicted by the partition concept pH. The data, therefore, strongly suggest that urinary pH is a major determinant of urinary oxyphenbutazone levels in horses.

The data reported here also shed light on the problem of "masking" (1). Masking is thought to occur when high levels of oxyphenbutazone in a urine sample interferes with the detection of other drugs. One of the principal objections to the use of phenylbutazone in racing horses is that horsemen can use it to mask the presence of other drugs. However, these data show no evidence for high blood levels of oxyphenbutazone, as might be expected if horsemen were attempting to cause masking problems. Rather, these results suggest that high urinary concentrations of oxyphenbutazone are found in horses forming alkaline urine, in which ionized oxyphenbutazone is trapped. High urinary concentrations of oxyphenbutazone are therefore more likely caused randomly by the physiology of the horse than by any ability or intent of horsemen to "load" urines with high or masking levels of oxyphenbutazone.

These observations are forensically important, since many racing jurisdictions regulate the use of phenylbutazone by measuring urinary levels of phenylbutazone and its metabolites. Most commonly, racing authorities have rules which state that medication with phenylbutazone within 24 or 48 hr of race time is not permitted. Some authorities enforce these rules based on the assumption that urinary levels of phenylbutazone and its metabolites are directly related to the time of administration of the last dose of phenylbutazone. In support of such rules, pharmacokinetic experiments on small numbers of horses (usually six or less) have been performed and reported in the literature (6) and elsewhere (7, 8). However, no investigators have studied the urinary kinetics of phenylbutazone and its metabolites over the full range of physiological pH values of post-race equine urines. Because of this, the validity of previous studies as a basis for regulatory and forensic decisions on racing horses is at best questionable.

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