

BRIEF COMMUNICATIONS

Values of urine specific gravity for Thoroughbred horses treated with furosemide prior to racing compared with untreated horses

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Abstract. The distribution of specific gravity values for 2,599 urine samples collected from racing Thoroughbred horses that were known to have received furosemide prior to racing was compared with that for 1,669 urine samples from racing Thoroughbred horses that reportedly had not received furosemide. Values of specific gravity for furosemide-treated horses were significantly lower ($P < 0.001$) than those for horses that had not received furosemide, and the proportion of horses with urine specific gravity either <1.010 or <1.012 was significantly greater ($P < 0.001$) among the furosemide-treated horses. These data indicate that evaluation of urine specific gravity would be a useful component of drug testing programs for regulation of furosemide use.

Furosemide (Lasix®) is the most widely used diuretic drug in performance horses in North America. Principally, it is used to prevent or abet exercise-induced pulmonary hemorrhage, although it has other therapeutic indications.⁴ The widespread use of furosemide in the racing industry has heightened concerns that because of its diuretic effects it may interfere with detection of other drugs by diluting their urinary concentration. Dilution of urine can lower illegal drug concentrations enough to render them undetectable by most routine screening methods.^{2,6,11,12} The effects of furosemide are relatively short lived, principally because the majority of intravenously administered furosemide is eliminated unchanged in the urine within 4 hours.⁴ Furosemide must be present at the luminal membrane of the renal tubule to exert its diuretic effect. Thus, the administration of furosemide within 4 hours of racing may affect concentrations of any illegal drugs.⁴

Furosemide reduces the urinary concentration of a number of illicit medications.^{2,4,6,11} For this reason, many jurisdictions regulate the use of furosemide in the racehorse.¹² These regulations, however, differ markedly among racing jurisdictions in North America. For example, time of treatment differs among jurisdictions from 2.5 to 5 hours before race time. Some racing authorities have attempted to control furosemide use by monitoring postrace samples of urine, blood, or both. Again, these regulations differ among jurisdictions, ranging from 50 to 100 ng/ml of furosemide in plasma or serum as a cutoff point (i.e., threshold value) for violation. Moreover, some jurisdictions (e.g., Texas) couple urine specific gravity values with serum furosemide concentrations. The rationale for coupling these 2 values is that because of the short-lived effects of furosemide on urine specific gravity, horses with high concentration of furose-

mid and low urine specific gravity (i.e., dilute urine) are more likely to be violators of furosemide regulations. For coupled testing, a cutoff point for urine specific gravity is selected, and horses with values below that point are subsequently tested for serum concentration of furosemide. An alternative strategy for coupled testing is to test both urine specific gravity and serum furosemide concentration for each horse sampled. Horses that are below the threshold concentration of specific gravity and in excess of the threshold concentration of serum furosemide are considered to be in violation of regulations. The validity of these various approaches to control use of furosemide, however, has not been systematically determined. Recent evidence suggests that tests for serum furosemide concentration alone lack accuracy as a method for identifying violators,¹ i.e., these tests will result in a relatively high number of horses falsely identified as being in violation of regulations (false violators). This lack of accuracy is attributable to considerable variation among horses with respect to serum concentration of drug as a function of time.¹ This variability appears to be present irrespective of the testing methodology used to detect furosemide.¹

The coupled testing of urine specific gravity and serum furosemide concentration has been experimentally determined to decrease the frequency of false violators (unpublished data). These experimental findings, however, were obtained from a small group of horses that had not been exercised. Although evidence is lacking that exercise alters pharmacokinetics of furosemide,³ exercise does alter urine specific gravity in some horses.⁸ Moreover, furosemide tends to enhance the magnitude of postexercise diuresis.⁹

The distribution of values for specific gravity of urine samples obtained after a race from horses that had or had not received prerace furosemide has not been reported. Thus, the purpose of this study was to determine whether the distribution of values of specific gravity from urine samples collected following racing differed between horses treated with furosemide prior to racing and horses that were not treated. The association between serum furosemide concen-

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tration and urine specific gravity among furosemide-treated horses also was examined.

Samples of urine and blood from horses racing in Texas are routinely submitted by the Texas Racing Commission veterinary staff to the Texas Veterinary Medical Diagnostic Laboratory (TVMDL, College Station, TX). All Thoroughbred horse samples submitted to the TVMDL from January 1, 1998, to February 1, 2001, were included in this study. Horses that had samples submitted to the TVMDL were selected at the racetrack on the basis of finish position (samples were submitted for all horses that finished first and second) or at the discretion of the veterinary staff of the Texas Racing Commission according to the following criteria: a horse finishing third in a race with a gross purse of \geq \$50,000, a beaten favorite, a horse selected at random by the stewards, or any other individual horse specified by the stewards or the Commission veterinarians. A midstream voided sample of the first postrace urination was collected by racetrack personnel in a designated area (test barn) at each track. Generally, urine was obtained within 2 hours (most often within 30 minutes) from the time of entry to the test barn. Venous blood samples (20 ml) were collected in clotting tubes immediately following collection of the urine sample. Venous blood samples were centrifuged to separate serum. Urine and serum samples were stored at 4 C until delivery to the TVMDL by racetrack security personnel to ensure an accurate chain of custody and to ensure that samples remained sealed until the time of analysis.

All laboratory testing was conducted in the Drug Testing Laboratory at the TVMDL. Urine specific gravities were determined using a digital urinometer.^a Because the lamp intensity of the urinometer was fixed, it was not possible to analyze turbid samples (whereas with a refractometer, the apparatus can be directed toward more intense light to obtain readings for most turbid samples). Samples that could not be analyzed by the urinometer because of excessive turbidity were tested by manual refractometry.^b

Serum furosemide concentrations were determined by high-pressure liquid chromatography (HPLC). For each sample, β -naphthoic acid^c (internal standard, 10 ng in 50 μ l ethanol^d), 200 mg $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$,^d and 6 ml ethyl acetate^d:HPLC grade acetonitrile^d (2:1) were added to 1 ml of serum. The samples were vigorously mixed for 20 seconds and then centrifuged at $1,080 \times g$ for 5 minutes. The organic supernatant was recovered and evaporated under nitrogen to dryness at 45 C. The residue was resuspended in 100 μ l acetonitrile:water (1:1). Furosemide levels were determined by injection of 35 μ l onto a reverse-phase column^e on an HPLC^f using a fluorescence detector to quantify furosemide and a photo diode array detector to confirm identity. (Fluorescence detector conditions: Ex/Em 231 nm/402 nm, 55 Hz, 2-second response time, pmtgain 13 at 9.5 minutes; change to Ex/Em 245/371, 220 Hz, 2-second response, pmtgain 13, total run time 12 min. Diode array detector conditions: 254 nm, 230 nm, and 280 nm with 4-nm bandwidth and reference wavelength at 450 nm with an 80-nm bandwidth. Solvent conditions: flow rate, 1 ml/min acetonitrile:0.4 N H_3PO_4 [40:60] for 11 minutes, followed by a single step gradient to acetonitrile:water [90:10] for approximately 1 minute. A 3-minute postrun equilibration with starting conditions was

completed prior to the next sampling to improve baseline stability.) Serum calibrators were prepared in quadruplicate by addition of appropriate volumes of furosemide^c and β -naphthoic acid to 1 ml of blank serum to produce concentrations of 80, 120, 160, and 200 ng/ml. The R^2 value for linear regression of the calibration curve was >0.996 . Quality control samples were prepared by addition of the appropriate amount of furosemide to obtain concentrations of 100, 140, and 180 ng/ml ($n = 3/\text{level}$). Within-day coefficient of variation (CV) estimates for the 100, 140, and 180 ng/ml concentrations ($n = 62$) were typically 4.7%, 7.1%, and 4.5%, respectively. Representative between-day CV estimates for the 100, 140, and 180 ng/ml concentrations were 11.1%, 9.4%, and 8.3%, respectively. Mean values were 107.5, 146.5, and 189.4 ng/ml for 100, 140, and 180 ng/ml ($n = 62$). The lower limit of quantification was 10 ng/ml ($s/n > 10$ with $<20\%$ variation from spiked value). The upper level of quantification was 300 ng/ml. All values >300 ng/ml were recorded as 300 ng/ml, and all values <10 ng/ml were recorded as 10 ng/ml for purposes of data analysis.

All urine samples included in the study were tested for furosemide by thin-layer chromatography (TLC) as previously described.¹⁰ The purpose of this test was to ensure that horses were correctly identified with regard to furosemide treatment. During the study period, 1 sample was excluded because the horse was not reported to have received furosemide but furosemide was detected in the urine sample by TLC, and 2 samples were excluded from analyses because furosemide was reportedly administered but none was detected in urine by TLC.

Data were recorded and maintained in a log book and a computerized database. The database included an identifier of the horse, date of sampling, racetrack, whether furosemide was administered, specific gravity of urine, and the concentration of furosemide in blood (when applicable). To maintain confidentiality, the horse identifier could be linked to the actual identity of the horse by only the Texas Racing Commission staff. Confidentiality of results was maintained, and results were only reported in the aggregate and without specifically identifying individual horses or owners.

The distributions of specific gravities of urine samples from horses treated with furosemide and from horses not treated with furosemide were represented graphically and using summary statistics (medians, interquartile ranges). The distribution of specific gravity values for furosemide-treated horses was compared with that for nontreated horses using the Wilcoxon rank-sum test.⁷ The prevalence of horses with values of urine specific gravity ≤ 1.010 and ≤ 1.012 were compared between furosemide-treated and nontreated horses using chi-square analysis.⁷ Comparison of these proportions also was stratified by year. Among horses treated with furosemide, the association of urine specific gravity (for each method) with serum furosemide concentration was examined graphically and using spline smoothing methods.⁵ For all statistical analyses, differences were considered significant at $P < 0.05$.

The distribution of specific gravity values for urine collected from horses following racing differed between horses that were treated with furosemide prior to racing and horses that were not treated with furosemide (Fig. 1). The median

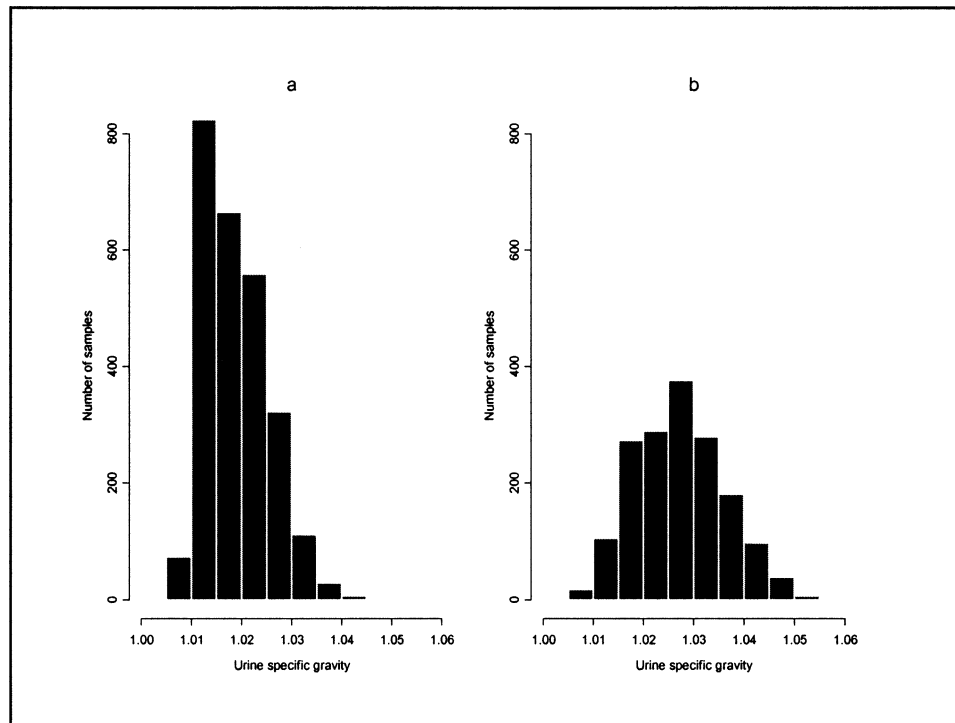


Figure 1. Histogram (frequency distribution) of specific gravity for 2,599 urine samples from horses treated prior to racing with furosemide (a) and 1,669 samples from horses that were not treated with furosemide prior to racing (b).

specific gravity of urine samples collected from furosemide-treated horses was 1.018 (interquartile range, 1.014–1.024), whereas that of samples collected from nontreated horses was 1.028 (interquartile range, 1.021–1.033). The mean and median were the same for the nontreated horses, but the mean for the furosemide-treated horses was 1.020, reflecting the skewing of these data (Fig. 1). The difference in urine specific gravity between groups was significant ($P < 0.001$). The proportion of horses with specific gravity values <1.010 was significantly greater ($P < 0.001$) among furosemide-treated horses (2.9%, 75/2,599) than among horses that were not treated with furosemide (1.1%, 19/1,669). Similarly, the proportion of horses with specific gravity values <1.012 was significantly greater ($P < 0.001$) among furosemide-treated horses (10.8%, 282/2,599) than among horses that were not treated with furosemide (2.3%, 39/1,669). There was no significant difference in urine specific gravity among years of the study, and specific gravity values were significantly lower ($P < 0.001$) among furosemide-treated horses than among nontreated horses during each year.

Among treated horses, values of specific gravity were plotted against the serum concentration of furosemide (Fig. 2). A spline smoothing plot was fitted to these data, revealing that at a value of approximately 1.025–1.030, furosemide concentration values tended to increase inversely with values of specific gravity. Additionally, several horses appeared to be outliers. Among furosemide-treated horses, 4 (0.2%) had a urine specific gravity of <1.010 and a serum furosemide concentration of >100 ng/dl; 19 (0.7%) horses had a urine

specific gravity of <1.012 and a serum furosemide concentration of >100 ng/dl.

Values of specific gravity were lower for urine samples collected following racing from horses that had been treated with furosemide prior to racing than for those from horses that were reported not to have been treated with furosemide. The proportion of horses with urine specific gravity values of <1.010 (a commonly used cutoff point for regulatory testing) was significantly greater among furosemide-treated horses than among nontreated horses. Moreover, the proportion of horses with specific gravity values of <1.010 or <1.012 among those not treated with furosemide was very small, indicating that exercise-induced diuresis would not be an important confounder for using postrace urine specific gravity testing to identify horses in violation of racing regulations. These findings indicate that urine specific gravity testing would be a useful component of testing procedures for regulating furosemide use. In light of experimental evidence indicating that testing serum furosemide concentration alone will result in a relatively high rate of false violators (i.e., horses falsely identified as having violated furosemide regulations),¹ racing jurisdictions that wish to reduce the number of false violators would benefit from coupling testing of urine specific gravity with testing of serum furosemide concentrations following racing. A specific gravity cutoff point of 1.012 would be better than 1.010 because the ratio of proportions of furosemide-treated and nontreated horses below the cutoff point was greater for specific gravity of 1.012 (10.8%/2.3% = 4.7) than for specific gravity of 1.010

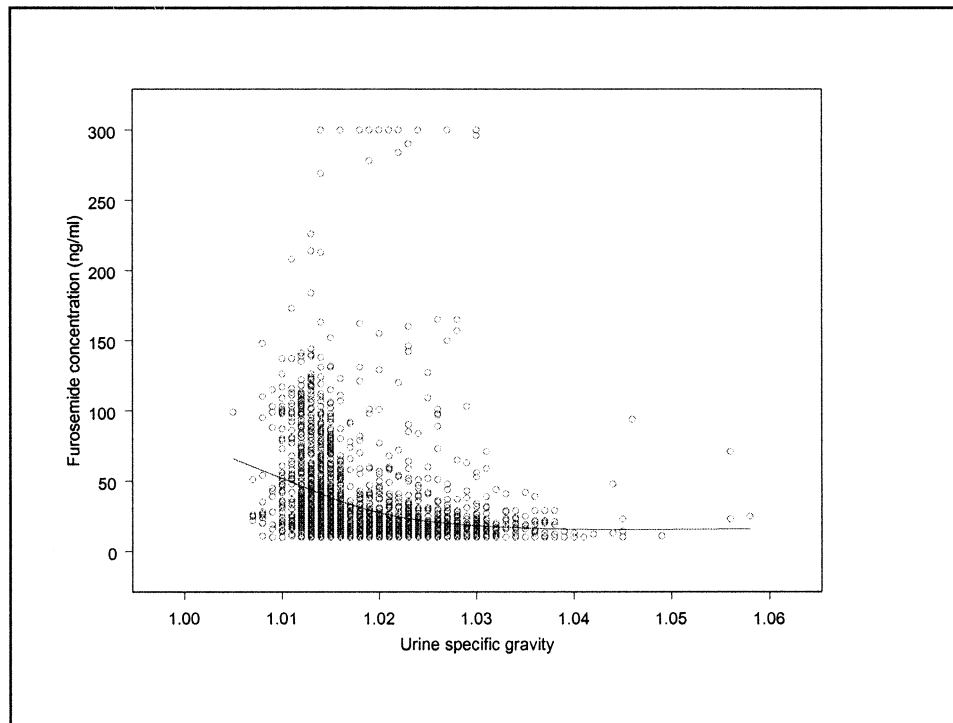


Figure 2. Scatter plot of serum furosemide concentration versus urine specific gravity for 2,599 samples from horses that were treated with furosemide prior to racing. The line represents a spline smoothing curve that was fit to the data.

(2.9%/1.1% = 2.6); thus, a cutoff point of 1.012 is almost twice as strong a discriminator. The higher cutoff point, however, also identified more samples (19 vs. 4) as being in violation of regulations.

Specific gravity values for furosemide-treated horses appeared skewed toward lower values (apparent log-normal distribution; Fig. 1). In contrast, the distribution of values of specific gravity for horses that were not treated appeared essentially normal (Gaussian). This difference in the shape of the histograms is consistent with prerace treatment with furosemide resulting in decreasing specific gravity of urine samples obtained following racing, thereby skewing the data for treated horses.

Although urine specific gravity values were significantly different between furosemide-treated and nontreated horses, the correlation between urine specific gravity and serum furosemide concentration among treated horses did not appear to be either linear or strong (Fig. 2). This finding likely reflects the fact that there is considerable interindividual variability in the pharmacokinetics of plasma furosemide¹ and the fact that plasma furosemide concentration and urine specific gravity are assessing related but distinct processes; plasma furosemide reflects the distribution and elimination of furosemide, whereas urine specific gravity reflects a physiologic effect of the drug on urine production. These data indicate that although urine specific gravity is significantly lowered by furosemide and that higher concentrations of serum furosemide tended to be found more commonly among horses with lower values of urine specific gravity, measure-

ment of urine specific gravity alone would not be a useful test for regulating furosemide use.

Plots of specific gravity versus serum concentration yielded other important information. For example, all but 1 of the values of furosemide concentration >250 ng/ml ($n = 22$ horses) were from samples collected from horses during the year 2000 (the exception was 1 horse in 1999), and all but 3 of the samples were collected at the same track (all samples >250 ng/ml at this track were collected during 2000). These discrepant findings by year and track would be of interest to racing regulatory officials. These outliers may reflect administration of furosemide to these horses by an alternative route (e.g., intramuscularly) or at an unapproved dose and time. Racing regulatory officials may find it helpful to graphically examine specific gravity values versus furosemide serum concentration among horses in their jurisdiction(s) to identify outliers, when such data are available. These outliers illustrate an important consideration of regulatory testing related to coupled testing. Testing that is conditional on specific gravity (i.e., testing specific gravity first, then testing the serum furosemide concentration) would miss outliers that represent apparent false compliers. Because identifying false compliers is of regulatory importance, joint testing of urine specific gravity and serum furosemide would seem preferable from a regulatory standpoint. Financially and technically, joint testing would be more demanding.

None of the horses with serum furosemide concentrations >250 ng/ml had urine specific gravities <1.012. This finding indicates that regulations for furosemide that rely solely

upon coupled testing of specific gravity and serum furosemide concentration may fail to detect some horses that may have violated regulations. As previously reported, raising the threshold concentration of plasma furosemide would be an effective strategy for reducing the rate of false violators of plasma furosemide regulations.¹ Raising the threshold alone, however, could be expected to increase the rate of false compliers. On the basis of the results of this and a previous study,¹ regulations that include both coupled testing of urine specific gravity and plasma or serum furosemide concentration and a threshold of furosemide concentration that is particularly high (e.g., 250 ng/ml) would improve the accuracy of furosemide testing.

There were limitations to this study. The presence of repeated observations for some horses (i.e., observations were not independent) was not accounted for in the analysis. Use of regression methods to account for this correlation or restricting the data to a single observation per horse, however, yielded results similar to those obtained by including all observations and assuming (incorrectly) independence. Given the large sample size and very small *P* values for comparisons, it is highly unlikely that failing to account for this correlation among samples would have altered the significance or direction of the observed associations. An assumption of this study was that horses were correctly identified with respect to exposure to furosemide. As described, all urine samples were tested for furosemide by TLC. Samples for which TLC results were discordant with furosemide status were excluded (3 samples). The net effect of such misclassification would have been to make the 2 groups more homogeneous and thereby to decrease the significant difference in values of specific gravity between furosemide-treated and nontreated horses. The time and dose of furosemide administered to the horses studied was not known. The extent to which this fact influenced results of this study could not be determined. The results of this study, however, reflect routine racetrack experience.

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Sources and manufacturers

- a. UroStat SG, StatSpin Inc., Norwood, MA.
- b. Atago URC-NE, Tokyo, Japan.
- c. Sigma Chemical Co., St. Louis, MO.
- d. EM Science, Darmstadt, Germany.
- e. Econosphere C18, Alltech Associates, Deerfield, IL.
- f. HP 1090 HPLC, Agilent Technologies, Palo Alto, CA.

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