# Estimation of the probability for exceeding a threshold concentration of furosemide at various intervals after intravenous administration in horses

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**Objective**—To estimate the probability for exceeding a threshold concentration of furosemide commonly used for regulatory purposes after IV administration of furosemide in horses.

**Animals**—12 mature healthy horses (6 Thoroughbreds and 6 Quarter Horses).

**Procedure**—Venous blood was collected from each horse prior to and 0.25, 0.5, 0.75, 1, 2, 3, 4, 4.5, 5, and 6 hours after administration of 250 or 500 mg of furosemide. Concentrations of furosemide were determined, using an ELISA. Concentration of furosemide was modeled as a function of time, accounting for inter- and intrahorse variabilities. On the basis of pharmacokinetic data, the probability for exceeding a concentration of 100 ng/ml as a function of time was determined, using a semiparametric smooth functional averaging method. A bootstrap approach was used to assess inherent variation in this estimated probability.

**Results**—The estimated probability of exceeding the threshold of 100 ng of furosemide/ml ranged from 11.6% at 4 hours to 2.2% at 5.5 hours after IV administration of 250 mg of furosemide/horse and 34.2% at 4 hours to 12.3% at 5.5 hours after IV administration of 500 mg of furosemide/horse. The probability of a horse being falsely identified in violation of regulatory concentrations was inversely associated with time and positively associated with dose.

**Conclusions and Clinical Relevance**—Interhorse variability with respect to pharmacokinetics of furosemide will result in misclassification of some horses as being in violation of regulatory concentrations. (*Am J Vet Res* 2001;62:320–325)

Furosemide is an organic acid loop diuretic commonly used in North America to prevent or control exercise-induced pulmonary hemorrhage in horses.[1] Because of its diuretic effects, furosemide may interfere with detection of other drugs by diluting their urinary concentration. Furosemide can reduce the urinary concentration of a number of drugs, including acepromazine, clenbuterol, theophylline, fentanyl, pentazocine, and morphine.[2-4]

This potential masking effect makes the widespread use of furosemide in horses involved in racing of considerable importance when considering regulation of medications administered to racehorses. Consequently, most racing jurisdictions regulate the administration of furosemide, and some monitor its use with postrace testing of selected race entrants.[5] Postrace testing often entails use of a threshold (cut-point) concentration in a serum sample collected shortly after completion of the race; the threshold is a concentration of furosemide that is interpreted in reference to the latest time at which furosemide was permitted to be administered (eg, 4 hours prior to racing). For many racing jurisdictions in North America, regulations are based on a threshold concentration of 100 ng of furosemide/ml of blood in samples obtained between 4 and 5.5 hours after IV administration of the drug.[5] Because of interhorse variability, when a specified threshold concentration is used after racing, a certain number of horses that have received furosemide in accordance with the regulations will be falsely classified as being in violation of the regulations (false-violators), and a certain number of horses that have received furosemide in violation of regulations will be falsely classified as nonviolators (false-compliers). Because of the regulatory implications, false-violators are of particular interest to regulatory officials and equine veterinarians. To the authors' knowledge, a systematic evaluation of the expected rate for exceeding the threshold concentration for violation of furosemide among horses given the drug in accordance with regulations (ie, expected rate of false-violators) has not been conducted. The purpose of the study reported here was to estimate the expected rate of false-violators for furosemide testing on the basis of statistical modeling of pharmacokinetic data, using specified values for the threshold concentration of furosemide and interval after administration. Results of the study would be clinically relevant for assessing the accuracy of programs that regulate furosemide violations on the basis of a cut-point for furosemide concentration. When an unacceptable rate of false-violators can be identified, it would be appropriate to modify such regulations.

## Materials and Methods

**Horses**—Twelve mature healthy mares (6 Thoroughbreds and 6 Quarter Horses) were used in the study. Body weight of horses ranged from 485 to 577 kg (median, 524 kg). Horses were housed at the Center for Equine Health, University of California, Davis and were members of a herd maintained for research and teaching purposes. These horses were maintained on a preventative medicine program that included regular deworming and vaccination. Horses were

Received Dec 7, 1999.
Accepted May 1, 2000.
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maintained in paddocks and fed grass hay and concentrate twice daily. Results of CBC and serum biochemical analyses were within reference ranges for each horse immediately prior to initiation of the study. The horses had not been used in any studies for 2 weeks prior to initiation of this study.

**Administration of furosemide**—Furosemide was administered IV to each horse, using 2 doses (250 mg/horse [approx 0.5 mg/kg of body weight] and 500 mg/horse [approx 1 mg/kg]). The rationale for examining these doses was that furosemide is routinely administered in these amounts to horses prior to racing. Separate experiments were conducted for each of the treatments, with a 2-week washout period between treatments. A fluoride-coated catheter was placed aseptically in a jugular vein of each horse. Furosemide was administered IV as a bolus via the catheter at 9 AM on each day of administration. Blood samples (13 ml) were collected via the IV catheter 0.5 and 0.25 hours prior to administration of furosemide and 0.25, 0.5, 0.75, 1, 2, 3, 4, 4.5, 5, and 6 hours after administration. The catheter was flushed with heparinized saline (0.9% NaCl) solution immediately after administration of furosemide and collection of each blood sample; the volume of heparinized saline administered was sufficient to flush and fill the catheter. Serum was separated from blood, using centrifugation, and stored frozen at –20 C until the time of analysis.

**Determination of furosemide concentration**—Concentration of furosemide in each serum sample was determined by use of a commercially available ELISA kit in accordance with the manufacturer's recommendations.[a] This assay is used as an official screening test for furosemide in various racing jurisdictions, including Kentucky, Maryland, and New York. The ELISA is a competitive inhibition assay. Briefly, 20 µl of standard, test, or control samples were added to each well along with 180 µl of furosemide-**horse radish peroxidase (HRP)** solution. All standard, test, or control samples were analyzed in triplicate. Free drug competitively prevents the antibody from binding to the furosemide-HRP conjugate. Therefore, the degree of antibody-furosemide-HRP binding is inversely proportional to the amount of drug in each sample. After incubation for 45 minutes, the liquid was removed from the microtiter wells, and the wells were washed 3 times (300 µl of wash buffer/wash). After another incubation (30 minutes), bound enzyme was detected by the addition of 150 µl of tetra methylbenzidine substrate solution. Quantitative test results were obtained by measuring and comparing the absorbance of wells of the samples against that of standards, using a microplate reader set at 650 nm. Results of ELISA were recorded in a computerized database and also were stored on computer diskettes. Data were electronically transferred to Texas A&M University for analysis.

**Statistical analysis**—Data were analyzed in 3 phases. First, we modeled furosemide pharmacokinetics (concentration of furosemide as a function of time) for each horse, in which the separate horse-specific coefficients accounted for subject-to-subject variability while the variance function described the within-subject variability, thereby taking into consideration inter- and intrahorse variabilities. Second, we evaluated the probability of exceeding a concentration of 100 ng/ml at various points in time, on the basis of the selected pharmacokinetic model and estimation method. Third, we used a bootstrap approach to properly assess variations in this estimated probability that resulted from the sample collection and estimation processes. These analyses were performed twice (once for the data obtained for each dose of furosemide [250 and 500 mg]).

For pharmacokinetic data, a heteroscedastic regression model[6] with nonlinear mean and variations behaving as a power of the mean was constructed to describe the concentration of furosemide in blood as a function of time. This model was represented by the following equation:

$$y_{ij} = f(x_{ij}, \beta_i) + \sigma (f[x_{ij}, \beta_i])^{\theta} \varepsilon_{ij}$$

where $y_{ij}$ denoted the jth (j = 1 to 10) concentration of furosemide determined by ELISA for horse i (i = 1 to 12), $x_{ij}$ was the jth sample collection time for the ith horse, $f(x_{ij}, \beta_i)$ represented the nonlinear mean function characterizing the relationship between $y_{ij}$ and $x_{ij}$, and $\beta_i$ represented the subject-specific regression coefficient in which between-horse variations were embedded (equation 1). The commonly used 1-compartment model, $\exp(\beta_{1i} - \exp[\beta_{2i}]x_{ij})$, was suitable for $f(x_{ij}, \beta_i)$, where $\beta_i = (\beta_{1i}, \beta_{2i})^t$ represented a vector of random coefficients. The element $f(x_{ij}, \beta_i)^{\theta}$ reflected the systematic variation within a subject. Additionally, $\sigma$ represented that component of the variability common to all subjects. Finally, $\varepsilon_{ij}$ denoted the random error with distribution of mean 0 and variance of 1. Validity of the mean and variance structure was assessed and confirmed by plotting the squared residuals and the variance function (ie, residual diagnostics). At the horse level, this subject-specific model of equation 1 was assumed for each horse. Additionally, a common model was constructed to describe variations among subject-specific regression coefficients. The combination of the 2 represented the population model in which samples obtained from the 12 horses were believed to be representative of the general population of Thoroughbreds and Quarter Horses. We used a **global 2-stage (GTS)** algorithm[6] to estimate coefficients in equation 1 as well as the between-subject variation. An expectation-maximization algorithm[7] was applied in the second stage of the GTS algorithm to obtain the population model. This approach accounted for between-subject and within-subject variations.

The study was conducted to calculate the probability ($P[x_0, y_{cutoff}]$) that the furosemide concentration (y) of a randomly chosen subject that received a specified dose of furosemide administered IV at time 0 would exceed a threshold concentration of furosemide ($y_{cutoff}$) at a specified period of time after injection ($x_{0\ hours\ after\ injection}$). Although $x_0$ should approximately be inside the range of the observed time period (0.25 to 6 hours), it need not be one of the time points in the original sample data. Values of ($P[x_0, y_{cutoff}]$) depend on point in time ($x_0$) and threshold ($y_{cutoff}$) and can be represented by the following equation:

$$P(x_0, y_{cutoff}) = \Pr(y \geq y_{cutoff}, \text{given that } x = x_0)$$

Using the previously described model for the pharmacokinetic (concentration versus time) data and estimated coefficients calculated by use of the GTS algorithm, a semiparametric smooth functional averaging approach was used to estimate the probabilities of exceeding the threshold concentration over time for a specified dose of furosemide.[b] For purposes of this study, we selected a threshold of 100 ng of furosemide/ml at times between 4 and 5.5 hours after IV administration of the drug, because it reflected common practices and guidelines for many racetracks in North America.

We then assessed variation of the resulting estimates, using a bootstrap method.[8] The rationale for this approach was that it provided statistical efficiency and flexibility. Five hundred bootstrap samples were generated on the basis of the original data without distributional assumptions placed on each coefficient of the pharmacokinetic model.[8] For each bootstrap sample, 12 pairs (emulating the original 12-horse data set) of coefficients were sampled with replacement from the original pool of estimates for each horse. Horse-specific estimates were used, because the true value of these coefficients was unknown, and the estimates provided the closest

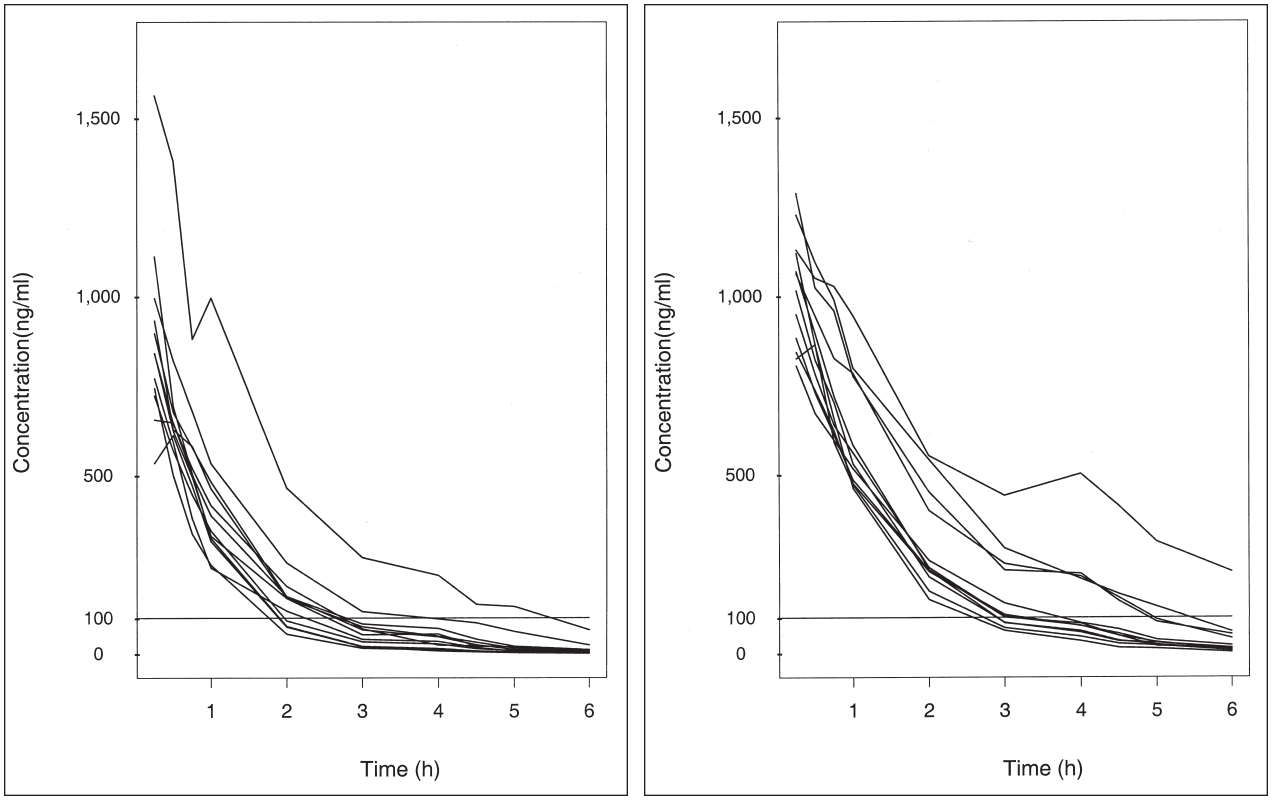


Figure 1—Plot of measured concentration of furosemide versus time for 12 horses that each received 250 (left) and 500 (right) mg of furosemide. Furosemide was administered IV at time 0. Each line represents data for 1 horse. The horizontal line represents a designated cut-point value of 100 ng/ml.

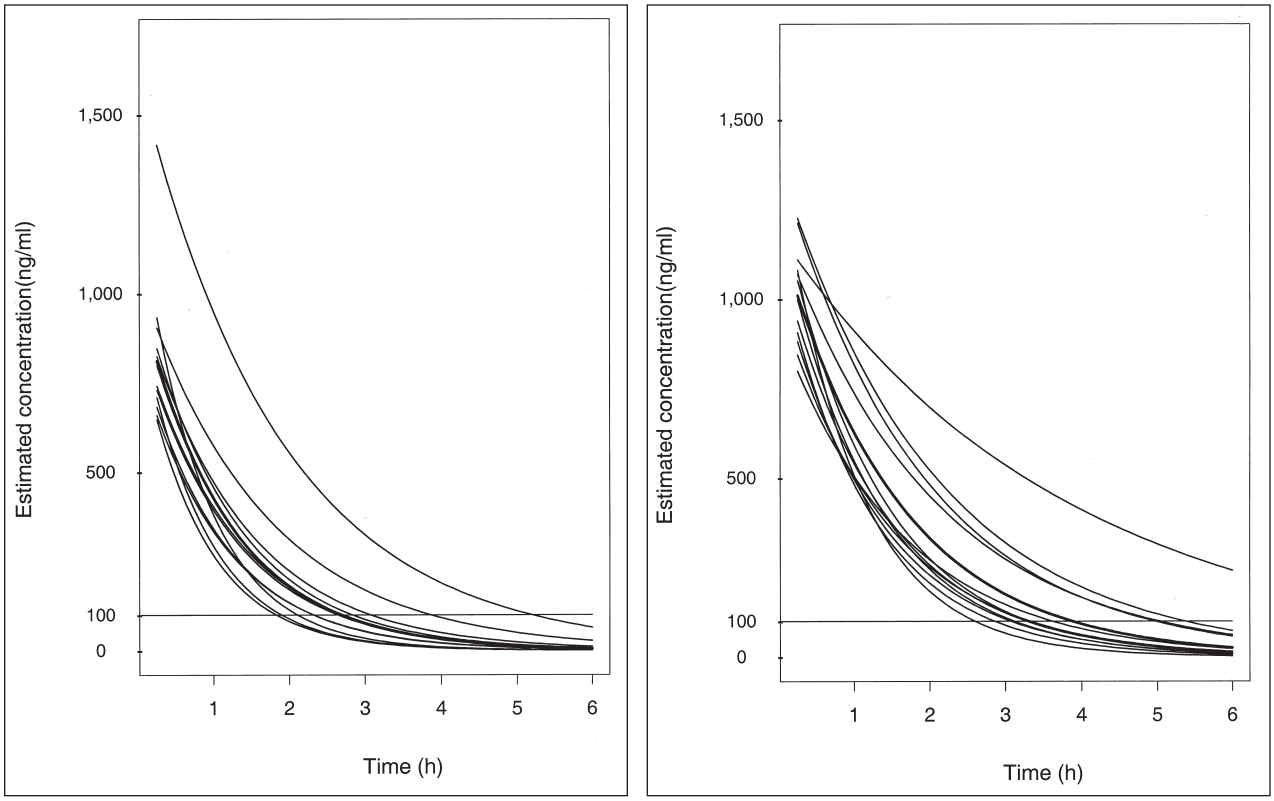


Figure 2—Plot of concentration of furosemide as a function of time, derived by using a 1-compartment model and global 2-stage algorithm regression methods, for 12 horses that received 250 (left) and 500 (right) mg of furosemide. *See* Figure 1 for key.

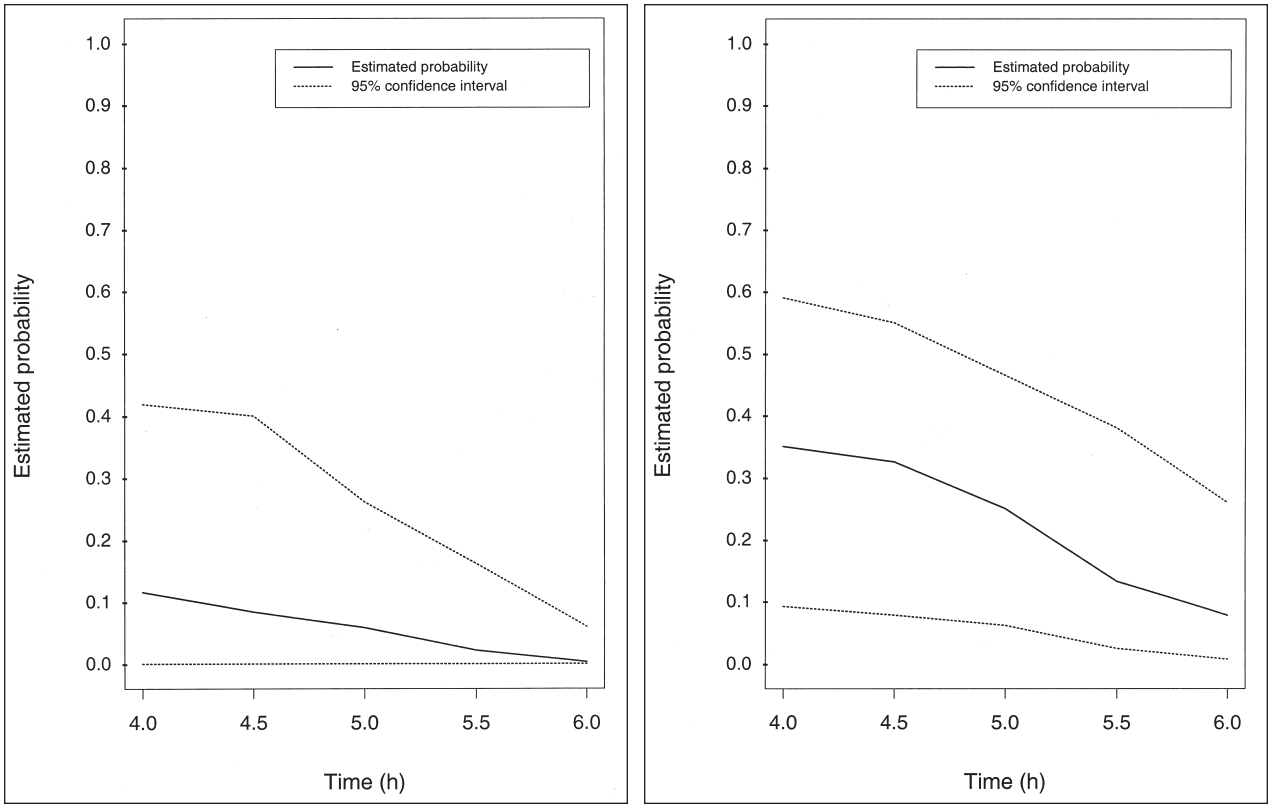


Figure 3—Estimates of the probability and 95% confidence interval for exceeding a concentration of 100 ng of furosemide/ml as a function of time after administration of 250 (left) and 500 (right) mg of furosemide to a horse. Furosemide was administered IV at time 0. Estimates were obtained by use of a semiparametric smooth functional averaging approach.

approximation. Pharmacokinetic data then were generated from equation 1, using the bootstrap-sampled coefficients. This procedure was repeated 500 times to obtain 500 bootstrap samples, each of which had 12 subjects. The semiparametric smooth functional averaging approach was applied to the bootstrap samples, and estimates of probabilities were evaluated along with their respective standard errors. To calculate the 95% confidence interval for the probabilities, we applied the adjusted percentile method, using the 500 bootstrap samples.[8] The predicted probabilities and their 95% confidence intervals for exceeding the threshold concentration were plotted against time.

The principal source of variation in these data was determined to be interhorse variability. Because the same quantity of furosemide (250 or 500 mg) was administered to each horse, body weight was considered as a possible cause of interhorse variability. To assess the effects of body weight, the Spearman rank correlation coefficient[9] was calculated for body weight with each of the 2 horse-specific coefficients from the pharmacokinetic model ($\beta_1$ and $\beta_2$ from equation 1) and with concentrations of furosemide at each of the sample collection times. Significance was defined as $P < 0.05$ for a 2-tailed test for these analyses.

## Results

Data from the 12 horses for each of the 2 doses of furosemide were plotted. For each horse, data points were connected (**Fig 1**). Analysis of these plots revealed considerable interhorse variability in pharmacokinetics of furosemide and indicated that horses with high plasma concentrations of furosemide tended to remain high, whereas, horses with low concentrations of furosemide tended to remain low. The GTS-estimated concentrations for each horse and for the population average were plotted for both doses of furosemide (**Fig 2**). For both doses, some horses were above the threshold concentration of furosemide of 100 ng/ml for a period ≥ 4.5 hours after IV administration of furosemide, although the mean for the population was below that threshold by 4.5 hours after administration of the drug.

Estimated probabilities for exceeding the threshold of 100 ng/ml ranged from 11.6% at 4 hours to 2.2% at 5.5 hours after IV administration of 250 mg of furosemide/horse and 34.2% at 4 hours to 12.3% at 5.5 hours after IV administration of 500 mg of furosemide/horse (**Fig 3**). The probability of a horse being a false-violator was inversely associated with time and positively associated with dose.

Significant correlations were not observed between body weight and either concentration of furosemide at any time point or coefficient of the pharmacokinetic model. Magnitude of correlation coefficients was weak, with none having an absolute value > 0.5 and most having an absolute value < 0.3.

## Discussion

In most horse racing jurisdictions, use of furosemide is regulated because of concerns that furosemide-induced diuresis may dilute the concentration of other medications used in an illicit manner. Regulation of furosemide often entails testing horses after racing to detect the concentration of furosemide in blood. For many racing jurisdictions, a threshold of 100 ng/ml when a horse received furosemide 4 hours

prior to racing is used to identify horses considered in violation of racing regulations regarding administration of furosemide.[10] Analysis of our results indicates that, on the basis of this threshold, a proportion of horses that have been administered furosemide in compliance with regulations will be falsely identified as being in violation of racing regulations. Probability for exceeding the threshold for violation decreased with decreased dose of furosemide and with increased time after administration of the drug. The problem of false-violators is of concern because violations of racing regulations generally carry penalties (fines and suspensions) levied against trainers or veterinarians.

The principal factor influencing the probability of a false-violator was the variability among horses (Fig 1 and 2). Horses that had higher concentrations of furosemide, as determined on the basis of results of an ELISA, in their initial sample continued to have high concentrations relative to other horses; moreover, horses that had higher concentrations of furosemide relative to other horses for the 500-mg dose were the same horses that had relatively higher concentrations for the 250-mg dose. We termed this phenomenon tracking.

To reduce the frequency of false-violators, the time after administration at which testing is performed could be extended (eg, collect blood for testing 7 hours after administration of furosemide) or the threshold (cut-point) concentration increased (eg, 200 ng/ml). Each of these solutions, however, would increase the rate of false-compliers (ie, horses given furosemide in violation of regulations but deemed to have been in compliance with regulations on the basis of results of furosemide testing). Because the principal concern regarding administration of furosemide to horses is its potential masking effect of illicitly administered medications, increasing the rate of false-compliers likely would be deemed undesirable by regulatory officials. The tracking phenomenon we observed indicates that it may be possible to identify horses that are likely to exceed the threshold of 100 ng/ml and that may require special consideration for drug-testing purposes. If it were possible to identify such horses in a simple and reliable manner (eg, testing serum prior to administration of furosemide to identify horses with a higher background concentration [ie, nonspecific reactivity]), strategies for modifying testing procedures could be implemented. Alternatively, coupling furosemide testing with another screening test (eg, urine specific gravity) may improve the accuracy of identifying horses that have received furosemide in violation of regulations.

Some racing jurisdictions use **high-pressure liquid chromatography (HPLC)**, rather than an ELISA, for detecting furosemide. Conceivably, results for estimates of specificity of testing may differ on the basis of the method of testing. Our findings, however, do not appear to be method-specific, because the blood samples tested by use of the ELISA in this study also were tested by use of HPLC with a mass-spectrometric detector and yielded virtually identical results with regard to between-horse variability and tracking (unpublished data). Thus, irrespective of the testing method used, there may be false-violators when serum is tested for concentration of furosemide between 4 and 5.5 hours after IV administration of a dose of 250 or 500 mg, using a threshold of 100 ng/ml.

Furosemide was administered as a fixed amount per horse, rather than on the basis of mg per kg of body weight. The rationale for this approach was that it was representative of the manner in which furosemide is administered to horses at racetracks. Body weight did not appear to account for all of the variability between horses that we observed. None of the pharmacokinetic variables examined was significantly correlated with body weight, and magnitude of the correlation coefficients was small. Horses that tended to have highest concentrations were not the horses that weighed the least. However, variation in body weight may have contributed to variability among horses for results of furosemide testing. The range of body weights in this study and the small sample size may have precluded our ability to detect a significant correlation between body weight and pharmacokinetic variables.

The small number of horses and the large inter-horse variability in the study reported here resulted in a lack of precision. Conducting this study with a larger number of horses would have made our confidence intervals more narrow, but we expect that our estimated probabilities for exceeding a specified threshold concentration would not have changed substantially.

This study involved the use of unexercised horses. The extent to which exercise influences the pharmacokinetics of furosemide has not been examined extensively. Submaximal exercise does not significantly alter pharmacokinetics of furosemide.[11] To the authors' knowledge, the extent to which a brief period of maximal exercise, such as during a race, would influence the pharmacokinetics of furosemide has not been examined. Effects of maximal exercise on decreases in renal blood flow may cause decreased clearance of furosemide, resulting in higher serum concentrations of furosemide. Alternatively, increased renal blood flow following maximal exercise may enhance renal clearance of furosemide. Additionally, contraction of plasma volume during maximal exercise may result in higher concentrations of furosemide.

Presently, regulations for administration and monitoring of furosemide vary among racing jurisdictions in North America, and uniform guidelines do not exist. Current recommendations may result in misclassification of some horses as being in violation of regulations, even though they have received a permissible dose of furosemide.

[a]Furosemide ELISA, Neogen Corp, Lexington, Ky.

[b]Chu K, Wang N, Stanley S, et al. A statistical evaluation of the regulation guidelines of furosemide usage (abstr), in *Proceedings*. Joint Stat Meet 1999;84.

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