

Estimation of the probability for exceeding thresholds of urine specific gravity and plasma concentration of furosemide at various intervals after intravenous administration of furosemide in horses

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Objective—To estimate the probability of concurrently exceeding thresholds for plasma concentration of furosemide and urine specific gravity after IV administration of furosemide in horses.

Animals—12 mature healthy Thoroughbred (n = 6) or Quarter Horse (6) mares.

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 IV administration of 250 mg (first experiment) or 500 mg (second experiment) of furosemide. Urine was collected hourly between 1 and 6 hours after administration of furosemide at both doses. Concentrations of furosemide were determined by use of an ELISA. Concentration of furosemide and urine specific gravity was modeled as a function of time, accounting for inter- and intrahorse variabilities. On the basis of pharmacokinetic and specific gravity data, the probability of exceeding a concentration of 100 ng of furosemide/ml as a function of time was determined, using a semiparametric smooth functional averaging method. A bootstrap approach was used to assess the inherent variation in this estimated probability.

Results—The estimated probability of exceeding the threshold of 100 ng of furosemide/ml and urine specific gravity < 1.012 was approximately 0% between 4.0 and 5.5 hours after IV administration of 250 mg of furosemide/horse, and ranged from 0 to 1% between 4 and 5.5 hours after IV administration of 500 mg of furosemide/horse. The probability of a horse being falsely identified as in violation of regulatory concentrations was inversely associated with time.

Conclusions and Clinical Relevance—Coupling plasma furosemide concentration with urine specific gravity testing will greatly reduce the chance that some horses are misclassified as being in violation of regulatory concentrations. (*Am J Vet Res* 2001;62:1349–1353)

Recently, we reported that the estimated rate of exceeding the threshold for violation of furosemide

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among horses in which regulations for administration of the drug were adhered to (ie, the expected rate of false-violators, or the number of horses that receive furosemide in accordance with regulations that are falsely classified as being in violation of regulations), using the cut-point of 100 ng of furosemide/ml of serum or plasma, was relatively high.¹ In an effort to improve the accuracy of testing and to reduce the rate of false-violators, some racing jurisdictions (eg, the Texas Racing Commission) couple plasma furosemide testing with measurement of urine specific gravity. Specifically, some racing jurisdictions determine urine specific gravity of selected race entrants immediately after racing. Any entrant with urine specific gravity below a specified cut-point (eg, 1.012) is subjected to postrace testing of plasma furosemide concentration. To our knowledge, the validity of this coupled approach has not been systematically evaluated. The purposes of the study reported here were to estimate the expected rate of false-violators of furosemide testing on the basis of statistical modeling of pharmacokinetic and urine specific gravity data, using specified values for the threshold concentration of furosemide (as previously reported¹), cut-point of specific gravity, and time, and to contrast the estimated rate of false-violators for the coupled testing strategy (ie, urine specific gravity and plasma concentration) with that of testing, using plasma concentration alone.

Materials and Methods

Horses—Twelve mature healthy Thoroughbred (n = 6) and Quarter Horse (6) mares were used; these were the same horses used in a previously reported study.¹ Body weight of horses ranged from 485 to 577 kg (median, 524 kg). 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.

Experimental protocol—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]), and blood samples were collected, as reported.¹ Concentration of furosemide in serum samples was determined by use of a commercially-available ELISA kit, according to the manufacturer's recommendations,² as described¹; these were the same furosemide concentrations as determined in a previously reported study.¹

Urine was collected from horses 30 minutes prior to administration of furosemide and hourly for 6 hours following administration. Urine was collected by catheterization of the urinary bladder. After aseptic preparation of the perineal

region, a sterile Foley catheter was placed in the urinary bladder. At each sampling time, the bladder was evacuated of urine by aspiration. After mixing, a 30-ml aliquot was collected. Urine was stored frozen at -20 C until measurement of specific gravity. Specific gravity was determined by use of a refractometer.^b The urinary specific gravity samples were simultaneously collected with the blood samples, when applicable.

Statistical analyses—Statistical analysis was conducted in 3 phases. First, we modeled furosemide concentration and specific gravity values 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. Next, we evaluated the probability of exceeding both testing thresholds (ie, a plasma furosemide concentration of 100 ng/ml and a urine specific gravity value < 1.012) at various points in time on the basis of the selected pharmacokinetic model and estimation method. Lastly, 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]).

A heteroscedastic regression model with nonlinear mean and variations behaving as a power of the mean was constructed, as previously described,¹ to describe the concentration of furosemide in blood as a function of time. This model was represented by the following equation:

$$y_{1ij} = f_1(x_{1ij}, \beta_{1i}) + \sigma_1(f[x_{1ij}, \beta_{1i}])^a \varepsilon_{1ij}$$

where y_{1ij} denoted the j th ($j = 1$ to 10) concentration of furosemide determined by ELISA for horse i ($i = 1$ to 12), x_{1ij} was the j th sample collection time for the i th horse, $f_1(x_{1ij}, \beta_{1i})$ represented the nonlinear mean function characterizing the relationship between y_{1ij} and x_{1ij} , and β_{1i} represented the subject-specific regression coefficient in which between-horse variations were embedded. The commonly used 1-compartment model, $\exp(\beta_{11i} - \exp[\beta_{12i}] x_{1ij})$, was suitable for $f_1(x_{1ij}, \beta_{1i})$, where $\beta_{1i} = (\beta_{11i}, \beta_{12i})'$ represented a vector of random coefficients. The element $f_1(x_{1ij}, \beta_{1i})^a$ reflected the systematic variation within a subject. Additionally, σ_1 represented that component of the variability common to all subjects. Finally, ε_{1ij} denoted the random error with distribution of mean 0 and variance of 1.

A homoscedastic regression model with nonlinear mean was constructed to describe the concentration of urine specific gravity as a function of time. This model was represented by the following equation:

$$y_{2ij} = f_2(x_{2ij}, \beta_{2i}) + \sigma_2 \varepsilon_{2ij}$$

where y_{2ij} denoted the j th ($j = 1$ to 6) value of urine specific gravity for horse i ($i = 1$ to 12), x_{2ij} was the j th sample collection time for the i th horse, $f_2(x_{2ij}, \beta_{2i})$ represented the nonlinear mean function characterizing the relationship between y_{2ij} and x_{2ij} , and β_{2i} represented the subject-specific regression coefficient, as β_{1i} in the first equation. The commonly used 2-parameter logistic model, $\beta_{21i}/(1 + \exp[\beta_{22i} \times \log x_{ij}])$, was suitable for $f_2(x_{2ij}, \beta_{2i})$, where $\beta_{2i} = (\beta_{21i}, \beta_{22i})'$ represented a vector of random coefficients. As σ_1 , σ_2 represented that component of the variability common to all subjects. Finally, ε_{2ij} denoted the random error with distribution of mean 0 and variance of 1.

For modeling both the plasma furosemide concentration data and urine specific gravity data, 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, the subject-specific models of the

2 equations were assumed for each horse. Additionally, a common model was constructed for the furosemide data and the urine specific gravity data to describe variations among subject-specific regression coefficients. The combination of the common model and the subject-specific model represented the population model. We used a **global 2-stage (GTS) algorithm**² to estimate coefficients in the 2 equations as well as the between-subject variations. An expectation-maximization algorithm³ 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 ($P(x_0, y_{\text{cutoff}1}, y_{\text{cutoff}2})$) that the furosemide concentration (y_1) and urine specific gravity (y_2) of a randomly chosen subject that received a specified dose of furosemide IV at time 0 would exceed both a threshold concentration of furosemide ($y_{\text{cutoff}1}$) and a threshold value of urine specific gravity ($y_{\text{cutoff}2}$) at a specified time after injection (x_0 hours after injection). It should be mentioned that although x_0 should approximately be inside the range of the observed time period (15 minutes to 6 hours), it does not need to be 1 of the time points in the original sample data. Values of ($P(x_0, y_{\text{cutoff}1}, y_{\text{cutoff}2})$) depend on the point in time (x_0) and thresholds ($y_{\text{cutoff}1}$ and $y_{\text{cutoff}2}$) and can be represented by the following equation:

$$P(x_0, y_{\text{cutoff}1}, y_{\text{cutoff}2}) = \Pr(y_1 \geq y_{\text{cutoff}1} \text{ and } y_2 \leq y_{\text{cutoff}2} \text{ given that } x = x_0)$$

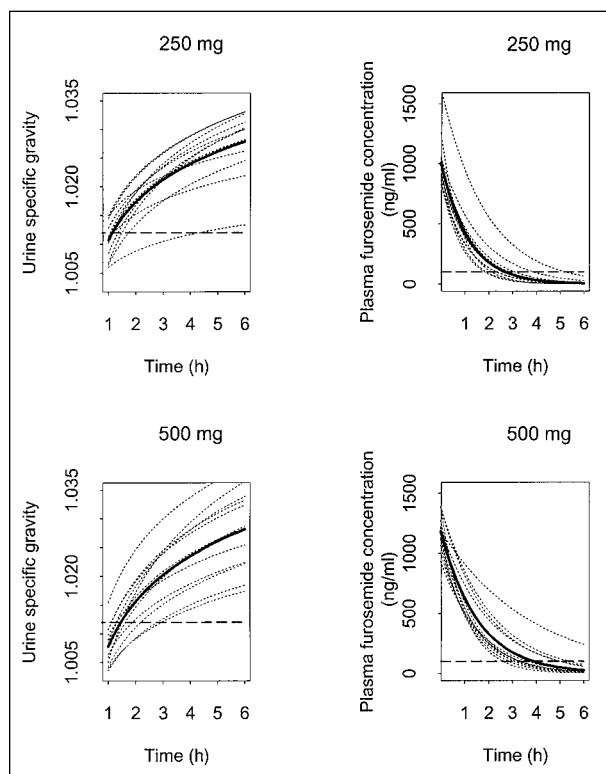


Figure 1—Plot of urine specific gravity (top left and bottom left) and plasma concentration of furosemide (top right and bottom right) as a function of time for 12 horses that each received 250 or 500 mg of furosemide IV at time 0; plasma furosemide concentration data were derived by use of a 1-compartment model and global 2-stage algorithm regression methods, and urine specific gravity were derived by use of a 2-parameter logistic regression model. Each small dashed line represents data for 1 horse; bold lines represent the population mean response. Horizontal large dashed lines represent the threshold (100 ng/ml for plasma furosemide concentration and 1.012 for urine specific gravity).

Using the previously described models in the first 2 equations with 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.⁴ For purposes of this study, we selected a threshold of 100 ng of furosemide/ml and a urine specific gravity threshold of 1.012 at times between 4.0 and 5.5 hours after IV administration of the drug, because they reflected regulatory guidelines for some racetracks in North America. The preadministration observations were not used for modeling the urine specific gravity data, because modeling the initial response to furosemide was not of interest, and only limited data (0.5 hours before and 1 hour after administration of furosemide) were available to assess this initial response.

We then assessed variation of the resulting estimated probabilities, using a bootstrap method.⁵ 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 the individual coefficient of the models.⁵ The exact procedure is described in an earlier publication.¹ We repeat the same process to generate pharmacokinetic data, using the first equation, and urine specific gravity data, using the second equation. 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 (CI) for the probabilities, we applied the adjusted percentile method, using the 500 bootstrap samples.⁵ The predicted probabilities and their 95% CI for exceeding the threshold concentration were plotted against time.

Results

The GTS-estimated concentrations for each horse and for the population average were determined for the 250-mg and 500-mg doses of furosemide, as reported.¹ The median value of urine specific gravity prior to

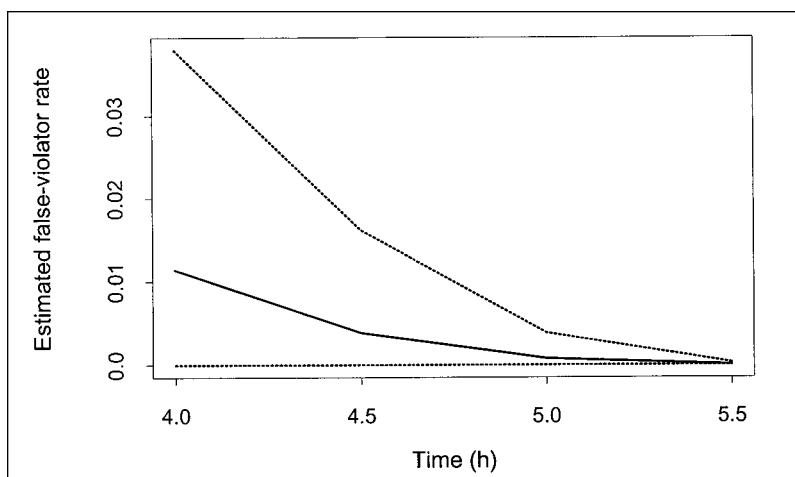


Figure 2—Probability (and 95% confidence interval) of falsely being in violation of racing regulations (ie, false-violators) when data from urine specific gravity (ie, value < 1.012) are coupled with plasma furosemide concentration (ie, exceeding a concentration of 100 ng/ml), as a function of time following administration of 500 mg of furosemide IV. Dashed lines represent the upper and lower bounds of the 95% confidence interval; solid line represents the estimated probability as a function of time.

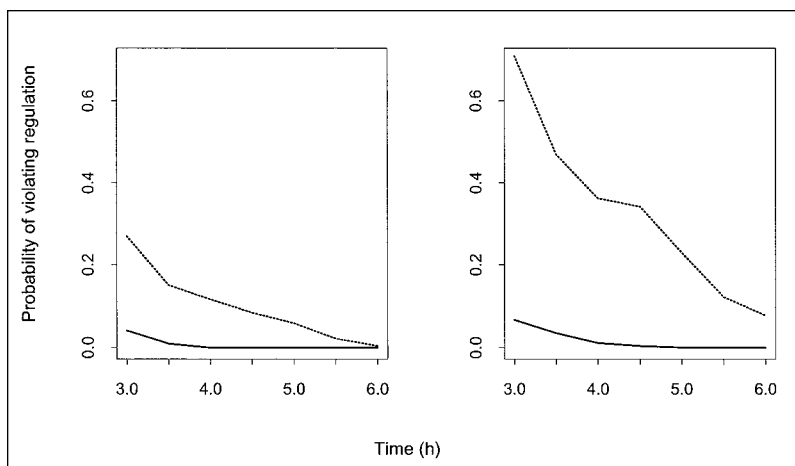


Figure 3—Probability of being in violation of racing regulations for either exceeding a plasma furosemide concentration of 100 ng/ml (plasma concentration test; dashed line) or having a urine specific gravity < 1.012 and exceeding a plasma furosemide concentration of 100 ng/ml (coupled testing strategy; solid line), as a function of time following administration of 250 mg (left) or 500 mg (right) of furosemide IV at time 0.

administration of furosemide was 1.028 (range, 1.004 to 1.054). Beginning 1 hour after administration, urine specific gravity increased over time (Fig 1). Although a large between-subject variability in urine specific gravity was observed, most horses had urine specific gravity values of > 1.012 approximately 2 hours after IV administration of 250 and 500 mg of furosemide. At each time point during the 6 hours, mean specific gravity and mean plasma furosemide concentration were negatively correlated (ie, were inversely associated). However, a horse's rank of specific gravity was not correlated with its rank for plasma furosemide concentration; for example, 1 horse consistently had the lowest values of specific gravity for the 250 mg dose. However, this horse did not have the highest plasma furosemide concentrations at this dose.

For the coupled testing strategy (ie, urine specific gravity < 1.012 and plasma furosemide concentration > 100 ng/ml), the estimated probabilities of violating regulations were calculated for both doses of furosemide; only the data for the 500 mg data were plotted (Fig 2), because the upper bound limit for the 95% CI was always $< 10^{-4}$ for the 250 mg data. The probability of a false-violator was inversely associated with time and positively associated with dose (Fig 3). Relative to use of plasma furosemide testing alone, the coupled testing protocol reduced the rate of false-violators to close to 0% at both doses of furosemide by 4.5 hours after administration.

Discussion

Results of this study are clinically relevant for assessing the accuracy of programs that regulate furosemide violation on the basis of coupling a cut-point of furosemide concentration and a cut-point of urine specific gravity and to assess the merit of coupled testing relative to plasma concentration testing alone. 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.⁹ Previously we reported that, on the basis of this threshold concentration, a high proportion of horses that were administered furosemide in compliance with regulations will be falsely identified as being in violation of racing regulations.¹ The probability of exceeding the threshold for violation decreased with decreased dose and with increased time after administration of the drug. Results of the present study indicated that coupling urine specific gravity testing with plasma furosemide testing would greatly reduce the rate of false-violators (Fig 3). This approach to regulating furosemide has been adopted by some racing jurisdictions (eg, Texas).

Coupling urine specific gravity with plasma furosemide concentration entails selecting cut-points for plasma furosemide concentration and urine specific gravity. As mentioned, a cut-point concentration of furosemide of 100 ng/ml is widely used. For urine specific gravity, the Texas Racing Commission uses a cut-point value of 1.012. Using these cut-points at the testing times of 4 to 5.5 hours after administration of furosemide, a testing protocol that coupled urine spe-

cific gravity with plasma furosemide concentration yielded an estimated false-violator rate of approximately 0% for the 250-mg dose and ranged from 0 to 1% for the 500-mg dose (Fig 2). Lowering the urine specific gravity cut-point to 1.010 resulted in estimated false-violator rates of 0% for both doses between 4.0 and 5.5 hours after administration of furosemide. This finding was not surprising because all but 1 horse had urine specific gravity values > 1.012 and plasma furosemide concentrations < 100 ng/ml during this period.

Values of urine specific gravity varied among horses and by dose of furosemide administered. One horse had a lower urine specific gravity than did other horses, irrespective of dose or time. Moreover, the specific gravity values for this horse were lower at the lower (ie, 250 mg) dose. At 4.5 hours after administration of furosemide, this horse would have been classified falsely as a violator after receiving 250-mg of furosemide, whereas it would have been correctly classified as a complier after receiving 500-mg. Intuitively, a larger dose of furosemide should have resulted in a smaller urine specific gravity reading. The paradoxical findings for this horse may be explained by the fact that some horses have lower values of urine specific gravity (irrespective of effects of medication), as well as the larger variability among subjects that may be associated with a larger dose of furosemide. Excluding the 1 horse, the variability in urine specific gravity values was smaller for the 250 mg dose than for the 500 mg dose; the overall population mean structures of urine specific gravity values appeared to be similar for both doses (whether data from that horse were excluded). Thus, a larger dose of furosemide may have amplified the variability among individuals that existed in the sample, thereby diminishing the influence of the 1 horse as an outlier with respect to urine specific gravity.

Although the urine specific gravity varied by dose, testing that coupled urine specific gravity with plasma furosemide concentration appeared to be robust with respect to the effects of this variability and the apparent outlier. Excluding the outlier from analysis of the 250 mg data set to estimate the rate of false-violators when coupling furosemide and urine specific gravity testing did not significantly differ from results obtained when data from that horse were included; 4, 4.5, 5, and 5.5 hours following administration of furosemide, the false-violator rate was 0%.

A crucial aspect of the coupled testing procedure is its dual usage of the 2 marginal procedures (ie, urine specific gravity and plasma furosemide concentration). The improved performance of this testing protocol with respect to reduction or elimination of false-violators may result from coupling 2 processes that measure different but related factors. Plasma furosemide concentration will be influenced by those factors determining the disposition, distribution, and elimination of the drug. Among horses that receive furosemide, urine specific gravity will be influenced by the diuretic effects of the drug and the physiologic responses of the host to that diuresis. Both procedures will be influenced by measurement error and inherent inter- and intraindividual variation.

Furosemide was administered at a fixed amount per horse, rather than on the basis of milligram per kilogram of body weight, to represent the manner in which it is administered to horses at racetracks. Neither pharmacokinetic variables nor urine specific gravity was significantly correlated with body weight, and the magnitude of correlation coefficients was small. The horses that had the highest concentrations or lowest urine specific gravities were not the horses that weighed the least. However, variation in body weight may contribute to variability among horses for results of furosemide testing. The limited 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 plasma furosemide concentration or urine specific gravity.

The small number of horses studied and the large interindividual variability resulted in a lack of precision. Performing this study with a larger number of horses would have made our confidence intervals narrower, but we expect that our estimated probabilities of exceeding the specified threshold would not be changed significantly.

This study was performed among horses that were not exercised. The influence of maximal exercise on urine specific gravity has not been well documented. Although urine specific gravity was reduced transiently following high intensity exercise,⁷ none of the horses in that report had a specific gravity < 1.012. Furosemide administration will enhance the magnitude of this postexercise diuresis.⁸ Although we did not examine the effects of maximal exercise, we do not believe that exercise would have altered our conclusion that coupling plasma furosemide concentration determinations with urine specific gravity determinations

would reduce the rate of false-violators, relative to determinations of plasma furosemide concentration alone.

Presently, regulations for administration and monitoring of furosemide vary among racing jurisdictions in North America. Current recommendations relying on measuring plasma concentration alone may result in misclassification of some horses that have received a permissible dose of furosemide as being in violation of regulations. Coupling urine specific gravity testing with plasma furosemide determinations will reduce the rate of false violations.

^bSchuco clinical refractometer, Fischer Scientific, Pittsburgh, Pa.

^cFurosemide ELISA, Neogen Corp, Lexington, Ky.

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