THE DETECTION, QUANTIFICATION AND PHARMACOKINETICS OF FUROSEMIDE AND ITS EFECTS ON URINARY SPECIFIC GRAVITY FOLLOWING INTRAVENOUS ADMINISTRATION TO HORSES

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Abstract

Furosemide is a potent loop diuretic used for the prevention of Exercise-Induced Pulmonary Hemorrhage in horses and may interfere with the detection of other substances by reducing urinary concentrations, so its use is strictly regulated. The regulation of furosemide in many racing jurisdictions is based on paired limits of urinary specific gravity of <1.010 and serum furosemide concentrations of >100 ng/ml.

To validate this regulatory mechanism, an LC-MS/MS method employing a solid phase extraction (SPE) procedure and furosemide-d5 as an internal standard was developed. The method was used to determine the pharmacokinetic parameters of furosemide in equine serum samples and its effects on urinary specific gravity after intravenous administration (250 mg) to 10 horses. Pharmacokinetic analysis showed that serum concentrations of furosemide were well described by a two-compartmental open model.

Introduction:

Furosemide (4-chloro-N-furfuryl-5-sulfamoylanthranilic acid; Salix®, Intervet, Millsboro, DE) is a potent organic acid loop diuretic used in North America for both control and prevention of Exercise-Induced Pulmonary Hemorrhage (EIPH) in horses[1]. Loop diuretics inhibit the active reabsorption of chloride ions in the thick ascending loop of Henle by binding to one of the Cl^- binding sites of the $Na^+/2Cl^-/K^+$ co-transport system, resulting in water retention in the tubule[2,3]. Since furosemide is a potent diuretic, it can interfere with the detection of other drugs or medications or their metabolites simply by diluting the urine, thereby reducing their concentrations.

Furosemide has also been reported to enhance the racing performance of horses[4,5]. As such, most racing jurisdictions seek to regulate the pre-race use of furosemide. The rules generally specify a dose of 250 mg per horse and administration by single intravenous administration no later than 4 h prior to post. A number of jurisdictions enforce these or similar regulations by evaluating post-race blood and urine samples. In this process, the urine sample is first screened to determine its specific gravity; if the specific gravity is less than a defined limit, commonly 1.010, then the concentration of furosemide in the corresponding serum or plasma sample is quantified. If that concentration is greater than a defined regulatory concentration, usually 100 ng/ml or thereabouts, an infraction of the furosemide rule is deemed to have occurred.

The regulatory thresholds incorporated into these rules are based on pharmacokinetic studies first reported from this group about 20 years ago[6], and collateral forensic experience. To more accurately define these thresholds, a highly specific and sensitive quantitative analytical method based on Liquid Chromatography/Mass Spectrometry/Mass Spectrometry for furosemide was developed. A pilot study was performed to re-define, under laboratory conditions, the pharmacokinetics of furosemide in the horse and to establish the relationships between serum furosemide concentration and urinary specific gravity at around four hours after IV administration of furosemide.

In developing the analytical method, a deuterated analog of furosemide was synthesized to serve as an internal standard. Then a solid phase extraction method and a highly sensitive quantitative LC-MS/MS method were developed for furosemide. Following furosemide administration, the pharmacokinetics and the relationship between dose, time after dosing, and serum concentrations of furosemide and urinary specific gravity were studied.

Materials and Methods:

Horses and Sample collection:

Ten mature Thoroughbred mares weighing between 542 and 572 kg were used for this study. All horses were acclimated to their stalls 24 h prior to experimentation. The animals were maintained on grass hay and feed (12% protein), which was a 50:50 mixture of oats and an alfalfa-based protein pellet. Horses were fed twice a day. The animals were vaccinated annually for tetanus and dewormed quarterly with ivermectin. A routine clinical examination was performed prior to each experiment to assure that these animals were healthy and sound. During experimentation, horses were provided water and hay *ad libitum*. All animal care was in compliance with the guidelines issued by the Division of Laboratory Animal Resources and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky.

Furosemide was administered as a single intravenous dose (250 mg) into the right jugular vein. Blood samples were collected from the opposite vein for analyses at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, and 24 h into Vacutainer serum tubes (Becton Dickinson, Rutherford, NJ), which were centrifuged at 4°C 800 ×g (2000 rpm) for 15 min. Separated serum samples were stored at -20°C until assayed. During the first day, complete urine collection was accomplished with a Foley catheter at 0, 1, 2, 3, 4, 5 and 6 h after administration. The 24 h sample was collected with a Harris flush tube (24 Fr × 152.4 cm; Seamless, Ocala, FL, USA). Urine was divided into appropriate aliquots and stored at –20°C until assayed.

Specific gravities were determined with a National refractometer (American Optical, Scientific Instrument Division, Buffalo, NY) calibrated to 1.000 with deionized water. The

specific gravity of each urine sample was quantified twice, and between each measurement the refractometer reading was checked with deionized water to ensure that no change in the control reading had occurred.

Synthesis of Furosemide-d5 Internal Standard:

The synthesis of furosemide-d5 is based on the method published in the patent literature[a]. Furosemide was prepared from 4-chloro-2-fluorobenzoic acid by chlorosulfonation and ammonolysis to the corresponding sulfonamide followed by reaction with furfurylamine. Later work by the same authors showed a similar, but more practical route using 2,4-dichlorobenzoic acid instead of 4-chloro-2-fluorobenzoic acid[7].

The five deuterium atoms were introduced into the furosemide molecule with the furfural fragment; the critical part of the synthesis was the preparation of furfurylamine-d5 (Figure 1, number 5). This was achieved as follows: direct cyanation[8] of furan-d4 (Figure 1, number 2) by chlorosulphonyl isocyanate in ethanonitrile at -78 °C, which provided, with moderate yield, the intermediate (2-furylcarbonyl-d3)sulphamoyl chloride (Figure 1, number 3), which, after addition of dimethylformamide (DMF), was converted into 2-furonitrile-d3 (Figure 1, number 4). The next step was reduction of the cyano group into an aminomethylene-d2 group by lithium aluminum deuteride, which resulted in introduction of the next two deuterium atoms in the furfural system and gave the desired furfurylamine-d5 (Figure 1, number 5). Deuterated furfurylamine was next reacted with 2,4-dichloro-5-sulfamoylbenzoic acid (Figure 1, number 6) using a modified procedure of Sturm *et al*[7]. As a result, furosemide-d5 (Figure 1,

[a] Sturm K, Siedel W, Weyer R (1962) US Patent 3,058,882

number 7) was obtained, and has been characterized using GC/MS and ^{1}H-NMR and found to be of high (>99%) chemical and isotopic purity.

Sample Preparation:

Standard solutions of furosemide (Sigma Chemical, St. Louis, MO) and furosemide-d5 (internal standard) were prepared in methanol. Extraction standards were prepared by the addition of a known volume of a furosemide solution (0, 5, 25, 50, 100, 250, 500 ng/ml) to blank serum samples. A known volume of a furosemide-d5 standard (10 µl of 10 µg/ml in methanol) was added to each sample, standard, and blank serum sample as an internal standard. The serum standards and blanks (1 ml/sample) were placed in culture tubes. The serum samples were acidified with 1 ml of 3% phosphoric acid in water.

Extraction Procedure:

The extraction procedure chosen was based on established Solid Phase Extraction (SPE) methodology. Clean Supelco DSC-18 Discovery columns (500 mg bed volume, Supelco, Bellefonte, PA, USA) were conditioned by washing sequentially with 1 ml of methanol, 1 ml of deionized water, and 1 ml of 1.5% (w/v) phosphoric acid. The columns were prevented from drying before applying specimens. Acidified serum samples (1 ml serum and 1 ml 3% phosphoric acid in water) were applied to the preconditioned columns. The columns were then washed consecutively with 1 ml of acetic acid solution (1 mol, pH: 5) and deionized water (1 ml). The columns were eluted with 2 ml of ethylacetate/dichloromethane/isopropanol/HCl (conc.) (65%/29%/5%/1%). The eluent was

evaporated to dryness under a stream of N_2 (<40°C) and, the dried eluents were dissolved in 75 µl of mobile phase consisting of 35% A:65% B, where A: acetonitrile, B: deionized water containing 1% triethylamine and 5% acetonitrile.

Instrumentation:

The instrument employed was a Hewlett-Packard Model 1050 LC attached to a Micromass VG Quattro-2 MS/MS in ESI tandem mode. The column was a Phenomenex Luna phenyl/hexyl 50 x 1.0mm x 3 micron phenyl-hexyl column. The mobile phase consisted of A: 35% / B: 65% where A: acetonitrile and B: deionized water containing 1% triethylamine and 5% acetonitrile. Each extract sample was transferred to an autosampler vial. A 7 µl aliquot was injected onto the LC-MS/MS. Detection involved negative mode electrospray ionization with acquisition of the following ions:

Chan Reaction	Dwell(secs)	Cone Volt.	Col.Energy
1 : 329.30 > 285.00	0.02	29.0	22.0
2 : 329.30 > 204.70	0.02	29.0	22.0
3 : 329.30 > 77.60	0.02	29.0	22.0
4 : 330.30 > 205.80	0.02	29.0	22.0
5 : 330.30 > 204.90	0.02	29.0	22.0
6 : 334.30 > 290.00	0.02	29.0	22.0
7 : 334.30 > 205.80	0.02	29.0	22.0
8 : 336.30 > 291.80	0.02	29.0	22.0
9 : 336.30 > 207.80	0.02	29.0	22.0

Those fragments arising from m/z 329.3 and 330.3 are specific to furosemide, whereas those arising from 334.3 and 336.3 are specific to the internal standard furosemide-d5. Quantitation was performed by calculating the ratio of the furosemide m/z 329.3->204.7 response relative to the internal standard m/z 334.3->205.8 transition.

Validation of the analytical method:

Linearity of the method was verified from the coefficient of determination (r^2) of the standard curves from six consecutive runs. Within-run accuracy and precision was determined by analyzing 6 replicate spiked samples at each of three concentrations (10, 100, and 400 ng/ml; Table 1a). The between-run accuracy and precision was determined by analyzing samples at these three concentrations in six consecutive runs (Table 1b).

The lower limit of detection (LOD) was calculated from six consecutive runs. The concentration calculated from the mean of the responses at zero concentration (y-intercepts) was determined. The LOD was defined as the concentration calculated from the mean response at zero concentration plus 2 standard deviations (the upper 95% confidence limit for zero)[9]. In addition to determining the LOD, an alternate calculation was performed utilizing the analyte's peak height compared to the baseline noise in the 329.3>204.7 m/z fragmentation chromatogram. By this method, the LOD was defined as the lowest concentration of analyte producing a peak greater than or equal to three times the baseline noise of the ion chromatogram.

The lower limit of quantitation (LOQ) was defined as the concentration calculated from the mean of the zero responses plus five times the standard deviation[9]. Extraction efficiencies were determined from six runs at three concentrations, 25, 100, and 250 ng/ml

(Table 1c). Each of the quantitative validation procedures included a concentration of 100 ng/ml, which is a widely used regulatory serum threshold concentration of furosemide.

Pharmacokinetics Analysis

Pharmacokinetic analyses were performed with a non-linear regression program (Winnonlin, version 3.1, Pharsight Corporation, Cary, NC). The goodness of the fit was evaluated by the Akaike Information Criterion (AIC), residual plots, and visual inspection. The data were weighted as $1/(y_{pred})^2$, where y_{pred} was the model-predicted concentration at the actual time. Area under the curve (AUC) following intravenous administration was measured by use of a linear trapezoidal approximation with extrapolation to infinity, and slope of the terminal portion (β) of the log serum drug concentrations versus time curve was determined by the method of least-squares regression[10].

The compartmental model used is represented by general equation *a* where Cp is plasma concentration of compound at any time (t), A and B are the Y intercepts associated with distribution and elimination phases, respectively, and α and β represent the rate constant of distribution and terminal elimination phase, respectively[11]. The rate constant of distribution (α), and distribution half-life ($t_{1/2}\,\alpha$) were determined using the method of residuals[12]. The terminal half-life ($t_{1/2}\,\beta$)[13] was calculated according to Equation 1.

$$Cp = A \times e^{-\alpha \times t} + B \times e^{-\beta \times t} \qquad (a)$$

$$t_{1/2}\beta = \ln 2/\beta \qquad (1)$$

Total body clearance (Cl_s) was calculated by use of Equation 2[14].

$$Cl_s = \text{IV Dose}/AUC_{0\text{-inf}}\,(\text{IV}). \quad (2)$$

The volume of distribution in central compartment (Vd_c), volume of distribution in terminal elimination phase (Vd_β), and volume of distribution at steady state (Vd_{ss}) were calculated according to Equations 3, 4 and 5, respectively[13].

$$Vd_c = \text{Dose (IV)}/A+B \quad (3)$$

$$Vd_\beta = \text{IV Dose} / AUC_{0\text{-inf}} \times \beta \quad (4)$$

$$Vd_{ss} = \text{IV Dose} \times AUMC_{0\text{-inf}} / (AUC_{0\text{-inf}})^2 \quad (5)$$

AUMC is area under the first moment curve and calculated by the trapezoidal method and extrapolated to infinity[10].

K_{10} is first order elimination rate constant, which describes elimination of drug from the central compartment. K_{12} and K_{21} are distribution rate constants from central to peripheral and from peripheral to central compartments, respectively. K_{10}, K_{12}, and K_{21} were calculated according to Equations 6, 7, 8, respectively.

$$K_{10} = \alpha \times \beta / K_{21} \quad (6)$$

$$K_{12} = \alpha + \beta - k_{21} - K_{10} \quad (7)$$

$$K_{21} = B\times\alpha+A\times\beta/(A+B) \quad (8)$$

Statistics:

Statistical analyses were performed by applying the central limit theorem of statistics to a sampling distribution of the sample mean in a *t*-test distribution with the single assumption that the low sample number is representative of the population in a binomial distribution. This allowed for the estimation of the probability for exceeding a threshold concentration of furosemide (100 ng/ml). For determination of the probability of exceeding specified values of serum furosemide concentration and urinary specific gravity, the SAS statistical program (Version 8.1, Cary, NC) was used. The frequency distributions of the serum furosemide concentrations and the urinary specific gravity concentrations at 4 h were analyzed for normalcy by using the Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling goodness of fit tests incorporated into this program.

Results:

The DSC-18 SPE-extracted 400 ng/ml total ion chromatographic (TIC) peaks of furosemide and furosemide-d5 were superimposable (Figure 2). Figures 3 and 4 show ion chromatograms of transitions specific to furosemide (from the TIC in Figure 2) and the ion chromatograms of transitions specific to furosemide-d5, also from the TIC in Figure 2, respectively.

The standard curve for the assay was linear from 5 ng/ml to 500 ng/ml, with a mean coefficient of determination r^2 of 0.9986 ± 0.00068 (n=6) (Figure 5). The within-run accuracy ranged between 93% and 99% for spiked samples at three concentrations. The precision was

determined by the coefficient of variation (CV) for the assay, which ranged from 1.4% to 15.3% (Table 1a). The between-run accuracy ranged between 100% and 104%, while the CV ranged from 3.5 to 12.8% (Table 1b).

The extraction efficiency, determined in three different concentrations, ranged between 96% and 100% with an expanded uncertainty range of 9.4 to 25% (Table 1c). Uncertainty was determined as described by the A2LA[b].

The LOD for furosemide by the LC-MS/MS method, calculated as suggested by Miller and Miller[9], was 1.8 ng/ml, and the LOQ was 3.9 ng/ml. Alternately, the LOD calculated from the S/N ratio of the 329.3>204.7 m/z fragmentation ion chromatogram was 2.2 ng/ml.

The urine specific gravity before furosemide administration (0 hour) ranged from 1.006 to 1.035 g/ml, with a mean of 1.023 g/ml ± 0.0037 (Figure 6). At 1 h after administration, the specific gravity dropped to 1.0093 g/ml ± 0.00073, and steadily increased thereafter through 6 h. Statistical analysis indicated that horses have less than an 85% chance at 1 h and less than a 0.05% chance at 2-6 h post-administration to have a urine specific gravity of <1.010 g/ml with the t-values increasing with subsequent time points. This suggests that there is less probability for a horse to have a urine specific gravity of <1.010 at later time points.

Serum furosemide concentrations declined rapidly after administration, presumably associated with the distribution of furosemide from the central compartment (Figure 7), and the relevant pharmacokinetic parameters are summarized in Table 2. The mean distribution half-life was 0.18 ± 0.004 h, and the mean terminal elimination half-life was 1.83 ± 0.134 h (Table 2). The terminal half-life was consistent between horses, ranging from 1.24-2.75 h, with a

mean of 1.83 h. The volumes of distribution at steady state and in the terminal phase were 0.363 L/kg and 1.48 L/kg, respectively, and the mean Vd_c was 169 ± 12 ml/kg. The systemic clearance was also closely distributed among the 10 horses, ranging from 0.324 L/kg/hr to 0.712 L/kg/hr, with a mean of 0.556 ± 0.039 L/kg/hr.

Since the current regulatory dose of furosemide is 250 mg administered intravenously 4 h prior to post, and regulation of the use of furosemide in many racing jurisdictions is based on a threshold concentration of 100 ng/ml in serum, it was estimated that the probability of exceeding the serum threshold from the distribution curve of serum furosemide concentrations at 4 hours post-administration. This analysis showed that serum furosemide concentrations at 4 hours post-administration are log normally distributed among the 10 horses studied, as shown in Figure 8 and results are summarized in Table 3. From this analysis it can be estimated that there is a less than 1/1,000 probability of exceeding a serum furosemide concentration of 31.8 ng/ml at 4 hours after 250 mg IV administration of furosemide.

At 4 h after administration, serum furosemide concentrations were log normally distributed (Figure 8). The probability of exceeding the serum threshold of 100 ng/ml was estimated from the distribution curve of serum furosemide (Table 3). Urine specific gravity values were normally distributed at 0, 1, and 4 h (Figure 9). The probability of exceeding the specific gravity threshold of 1.010 at 4 h after dosing was estimated from that distribution curve (Table 4). These analyses also show that the probability of a urine specific gravity value of 1.016 at 4 h post-administration is considerably less than 1 in 1,000.

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[b] Calculation of measurement uncertainty for type III methods. Interim A2LA policy on measurement uncertainty for type III methods. The Life Sciences Committee, American Association of Laboratory

Discussion:

Furosemide is a potent loop diuretic, commonly used in North America in the prevention of exercise-induced pulmonary hemorrhage (EIPH) in horses. As a diuretic, it may interfere with the detection of certain other drugs and drug metabolites by diluting the urine, thereby reducing urinary drug, medication, or metabolite concentrations. Therefore, its use is closely regulated in many racing jurisdictions.

Currently the regulation of furosemide is based on a two-step regulatory process. Samples are first screened for urinary specific gravity; if the urinary specific gravity is below a specified value, usually 1.010, then the concentration of furosemide in the serum is quantified. If the serum concentration is greater than a specified threshold, usually 100 ng/ml, an infraction of the furosemide medication rule may be deemed to have occurred

The original research which resulted in the establishment of this 100 ng/ml serum threshold for furosemide was done in our laboratory about 20 years ago[6,15,16]. In these studies, the kinetic parameters of furosemide were established which were best described by a three compartment open-body model, and the mean plasma concentration of furosemide after intravenous administration (0.5 mg/kg) at 4 h after dosing was about 9.6 ng/ml, with a standard deviation of about 3.1 ng/ml. The population distribution of these plasma furosemide values was log-normal, and, based on this population distribution, the statistical probability of identifying a plasma concentration >24.6 ng/ml at 4 h after dosing was estimated at less than 1

in 1,000. This regulatory threshold or limit was then rounded up to 30 ng/ml, and this value was suggested as the regulatory threshold for furosemide[6].

Practical aspects of equine forensics resulted in the modification of this regulatory threshold in two ways. First, because the principal regulatory concern with furosemide was its ability to dilute equine urine, determination of specific gravity was incorporated into the process. Based on analytical opinion, a regulatory limit of 1.010 for urinary specific gravity was selected as the screening "cut-off". Urinary specific gravity screening was rapid and inexpensive, and therefore allowed for highly cost-effective identification of urine samples in which significant dilution of illegal medications may have occurred.

Once the potential "problem" samples had been identified by specific gravity screening, the furosemide concentration in the matched serum samples was quantified. Then, if the concentration of furosemide in the sample was above the specified "cut-off", an infraction of the furosemide rule was deemed to have occurred and regulatory action could result.

The second modification to this protocol was an increase in the regulatory threshold for serum. The first state to implement this threshold approach was Oklahoma in the mid-1980's, and the Oklahoma authorities arbitrarily increased the regulatory threshold to 50 ng/ml. Then, as the potential regulatory value of this approach was realized, other states added the specific gravity screening step, and the regulatory limit was further increased, often to 100 ng/ml. Currently a number of states, including Kentucky, Ohio, Maryland and New York, regulate the use of furosemide in this way, using the 1.010 specific gravity "cut-off" in combination with a 100 ng/ml serum threshold for regulatory purposes.

The purpose of this study was to re-evaluate these regulatory thresholds, using newer and more accurate analytical and statistical techniques. In this regard, the first step was to

develop a highly sensitive LC-MS/MS method utilizing solid phase extraction (SPE) to determine the pharmacokinetic parameters and "detection times" for furosemide in equine serum samples after intravenous administration (250 mg) to help regulatory agencies control this agent in racing horses. Therefore, an LC-MS/MS method employing a solid phase extraction procedure to detect and quantify furosemide in serum samples of horses was developed with the LOD being 1.8 ng/ml, and the LOQ being 3.9 ng/ml.

Determination of the pharmacokinetic parameters of furosemide in racing horses provides important information to racing authorities. One objective of this study was to determine the serum furosemide levels, which might suggest compliance with a 4 h furosemide rule. The pharmacokinetic analysis used showed that furosemide is rapidly cleared following IV administration, with a mean terminal half-life of 1.8 h. Serum furosemide concentrations at 4 h post-administration ranged from 6.6-21.5 ng/ml with a mean concentration of 10.9 ng/ml ± 1.4 (SD) which was not significantly different from the previous study conducted 20 years ago[6]. Additionally, this study indicated that there is less than 0.05% chance for a serum furosemide level to exceed 100 ng/ml and for urine specific gravity to be less than 1.010 g/ml at 2 h and later post-administration. Three of the ten horses had serum furosemide concentration below our LOQ at 6 h. The concentrations of furosemide in the remaining seven horses at 6 h ranged from 5-9.5 ng/ml with a mean concentration of 6.8 ng/ml ± 0.66 (SEM).

By using the mean values of serum furosemide concentration and urine specific gravity, horses have both serum furosemide concentration greater than 100 ng/ml and urine specific gravity less than 1.010 at only one hour post-administration time and

Statistical analysis indicated that at 1 h post-administration, there are less than 85% and 75% of chances, respectively for horses to have a urine specific gravity <1.010 and serum

furosemide concentration >100 ng/ml. At later post-administration time points, these chances are <0.05% for both urine and serum samples to exceed those thresholds. Based on this study, it is very unlikely for horses to have serum furosemide concentrations >100 ng/ml or urine specific gravity <1.010 at 4 h post-administration (250 mg IV). On the other hand, it should be remembered that urine specific gravity is highly variable among horse, and even without furosemide administration horses might naturally have urine specific gravity <1.010.

The data developed in this study (Tables 3 and 4) can be used to develop a range of furosemide serum and urine specific gravity threshold levels. The diuretic activity of furosemide is rapid, occurring within 10-15 min, and is largely over within 1-3 h following IV administration.

1a. Within-run Accuracy and Precision

Concentration (ng/ml)	Measured concentration (ng/ml; mean ± SEM) (n=6)	Accuracy (%) (mean ± SEM)	C.V. (%)
10	9.31 ± 0.58	93.12 ± 5.81	15.3
100	98.63 ± 1.46	98.63 ± 1.46	3.63
400	397.83 ± 2.69	99.13 ±0.56	1.42

1b. Between-run Accuracy and Precision

Concentration (ng/ml)	Measured concentration (ng/ml; mean ± SEM) (n=6)	Accuracy (%) (mean ± SEM)	C.V. (%)
10	10.32 ± 0.53	100.32 ± 5.32	12.86
100	103 ± 30	103 ± 3.0	7.2
400	415 ± 5.78	104 ± 1.48	3.54

1c. Extraction Efficiency

Concentration (ng/ml)	Extraction efficiency (%) (mean ± SEM) (n=6)	C.V. (%)	Expanded Uncertainty (%)
25	96 ± 4.1	10.03	25
100	100.8 ± 1.85	4.54	11.4
250	100.4 ± 1.53	3.75	9.4

Table 1. Within-run and between-run assay accuracy and precision (a, b), extraction efficiency (c) of the LC-MS/MS assay used to quantify furosemide in horse serum samples (from top the bottom). Uncertainty was determined using the method of A2LA (2002). The mean ± the expanded uncertainty equals the 95% confidence range.

Horse	1	2	3	4	5	6	7	8	9	10	Mean ± SEM
Weight (kg)	556	572	554	542	564	542	564	588	590	602	567.4 ± 6.48
K_{10} (hr^{-1})	3.29	3.38	3.29	3.07	3.51	3.55	3.15	3.65	3.13	3.04	3.31 ± 0.067
α (hr^{-1})	3.97	4.04	3.69	3.71	4.06	4.16	3.65	4.24	3.73	3.59	3.88 ± 0.074
β (hr^{-1})	0.396	0.382	0.386	0.426	0.436	0.557	0.309	0.462	0.252	0.345	0.395 ± 0.0267
$t_{1/2}\alpha$ (hr)	0.175	0.171	0.188	0.187	0.171	0.167	0.189	0.164	0.186	0.193	0.179 ± 0.0033
$t_{1/2}\beta$ (hr)	1.75	1.82	1.79	1.63	1.59	1.24	2.24	1.50	2.75	2.0	1.83 ± 0.134
Cl_s (L/kg/hr)	0.324	0.474	0.517	0.560	0.679	0.444	0.625	0.712	0.551	0.676	0.556 ± 0.039
Vd_c (ml/kg)	99	140	157	183	193	125	198	195	177	222	168.9 ± 12.01
Vd_β (L/kg)	0.819	1.24	1.34	1.32	1.56	0.798	2.02	1.54	2.19	1.96	1.48 ± 0.151
Vd_{ss} (L/kg)	0.223	0.320	0.287	0.377	0.378	0.223	0.450	0.382	0.502	0.488	0.363 ± 0.032
R^2	0.999	0.995	0.994	0.991	0.999	0.997	0.995	0.997	0.993	0.996	0.996 ± 0.0008

Table 2. Pharmacokinetic parameters of furosemide following single 250 mg IV administration (n=10).

Probability of exceeding the value (%)	Serum concentrations of furosemide (ng/ml)
50	10.21
25	13.08
10	16.35
5	18.69
1	24.01
0.1	31.79

Table 3. Probability of exceeding the indicated serum furosemide values at four hours post-administration time following 250 mg IV administration of furosemide based on 10 horses included in our kinetics study.

Probability of exceeding the value (%)	Urine Specific Gravity
50	1.0093
25	1.0109
10	1.0123
5	1.013
1	1.015
0.1	1.016

Table 4. Probability of exceeding the indicated urine specific gravity values at four hours post-administration time following 250 mg IV administration of furosemide based on 10 horses included in our kinetics study.

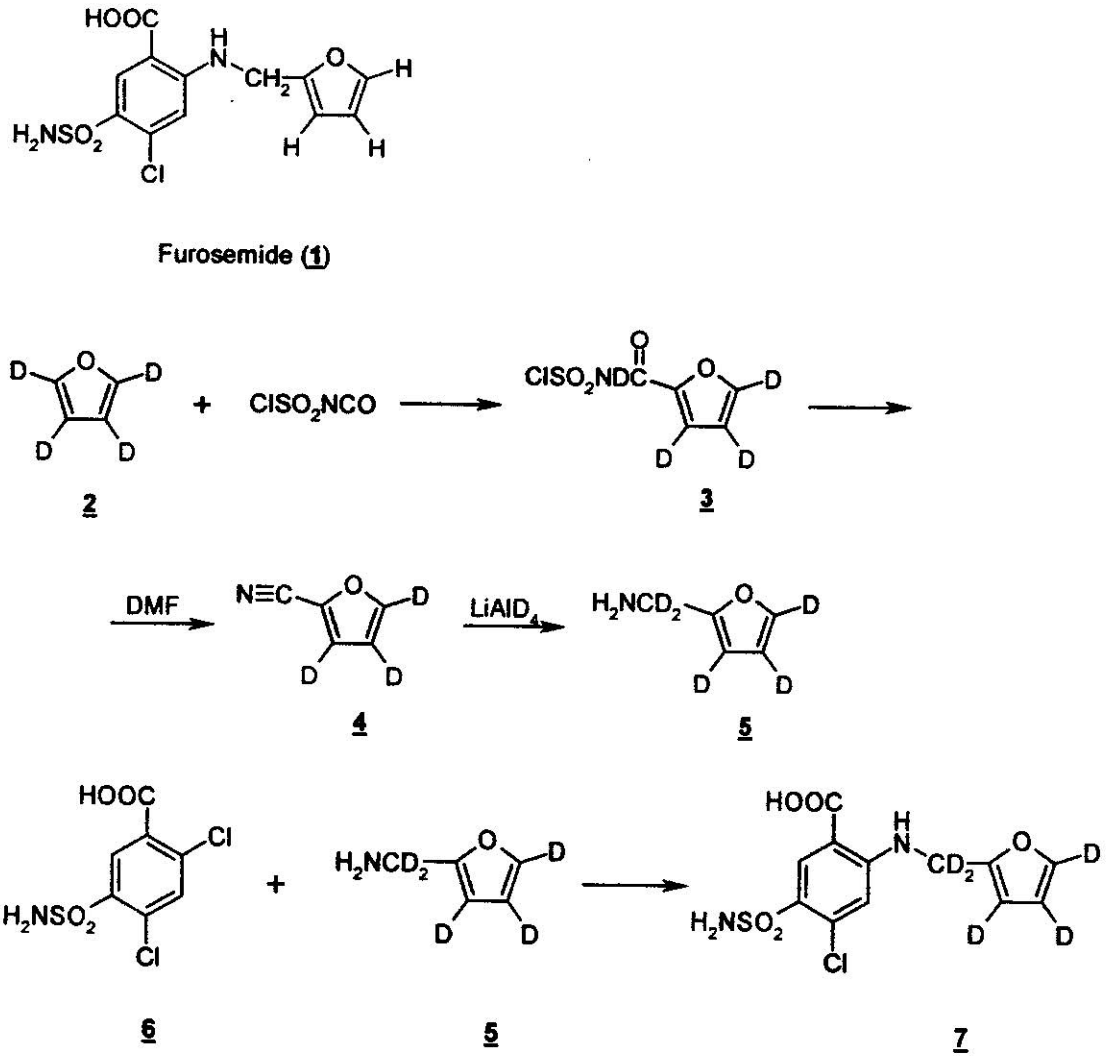

Figure 1. Synthesis of furosemide-d5 as an internal standard.

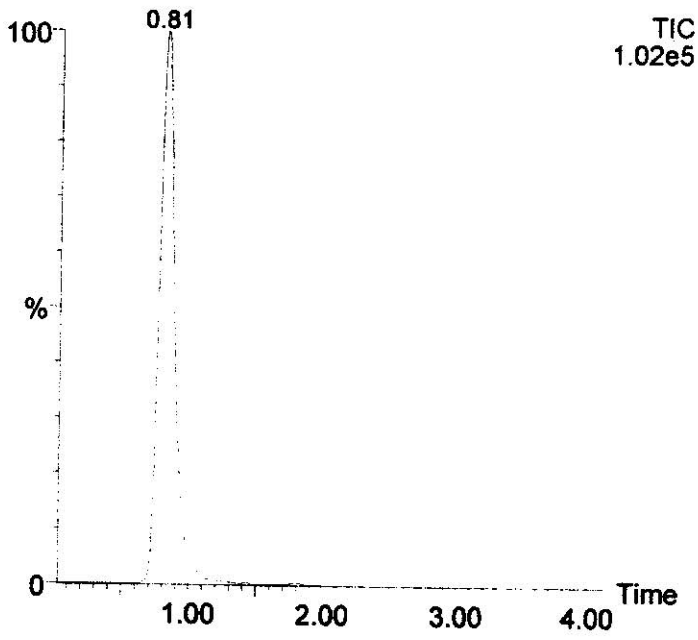

Figure 2. Example of furosemide chromatography. DSC-18 SPE extracted 400 ng/ml furosemide calibrator. TIC of furosemide and furosemide-d5 is shown, with both peaks precisely overlapping.

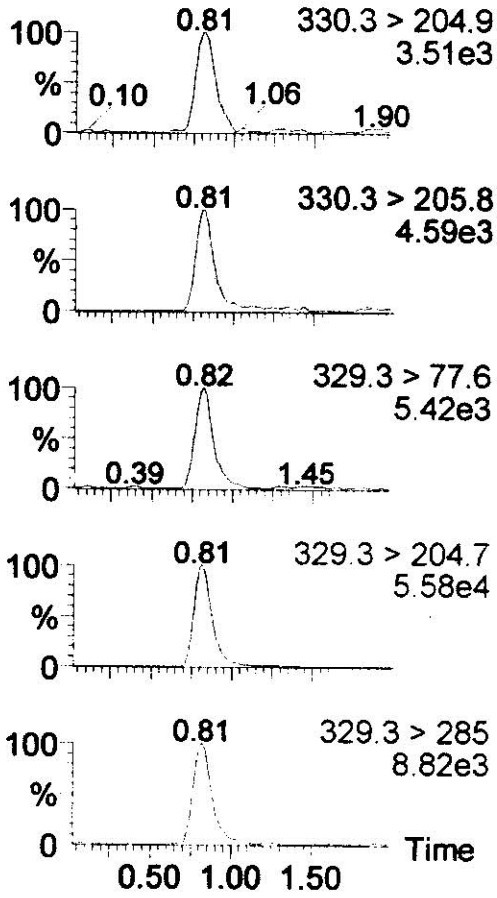

Figure 3. Ion chromatograms of transitions specific to furosemide (from Figure 1 TIC)

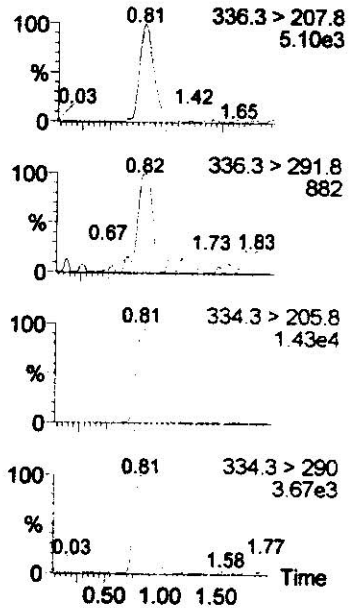

Figure 4. Ion chromatograms of transitions specific to furosemide-d5 (from Fig. 1 TIC)

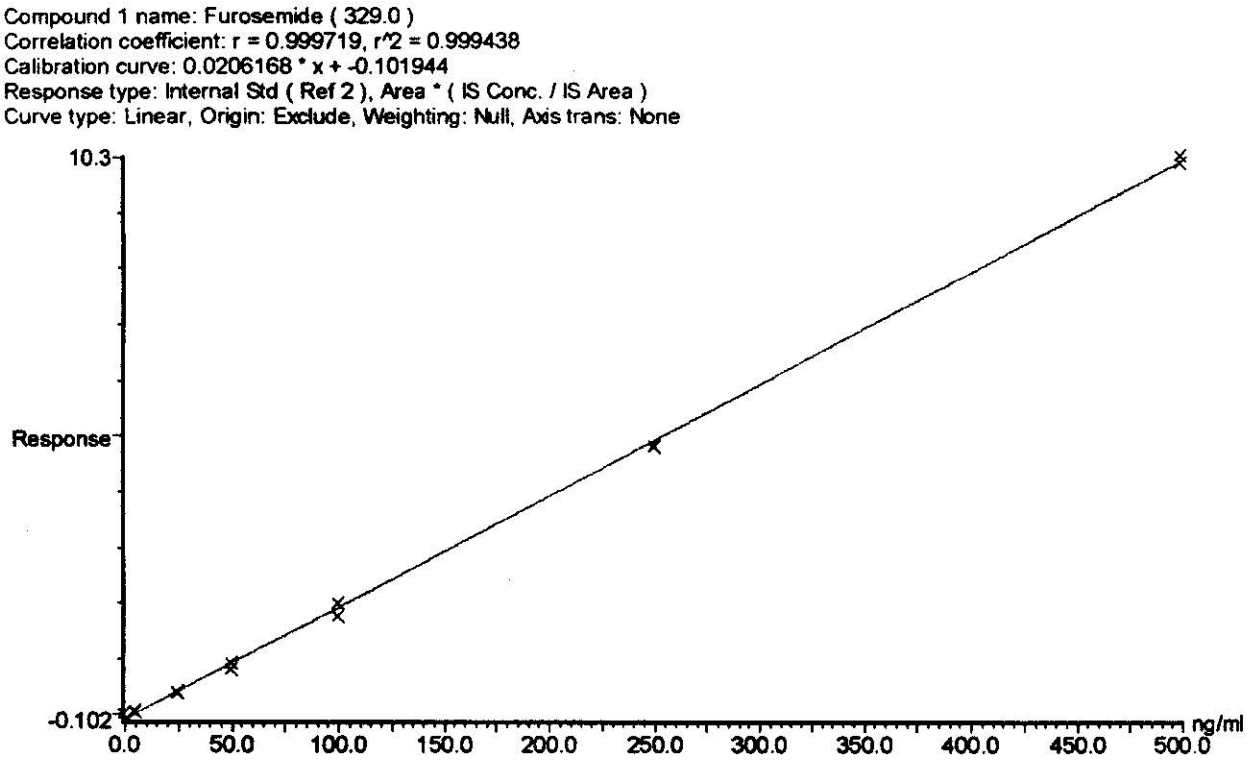

Figure 5. Typical calibration curve generated by MassLynx 3.4 software, indicating coefficient of determination (r^2) of 0.999438.

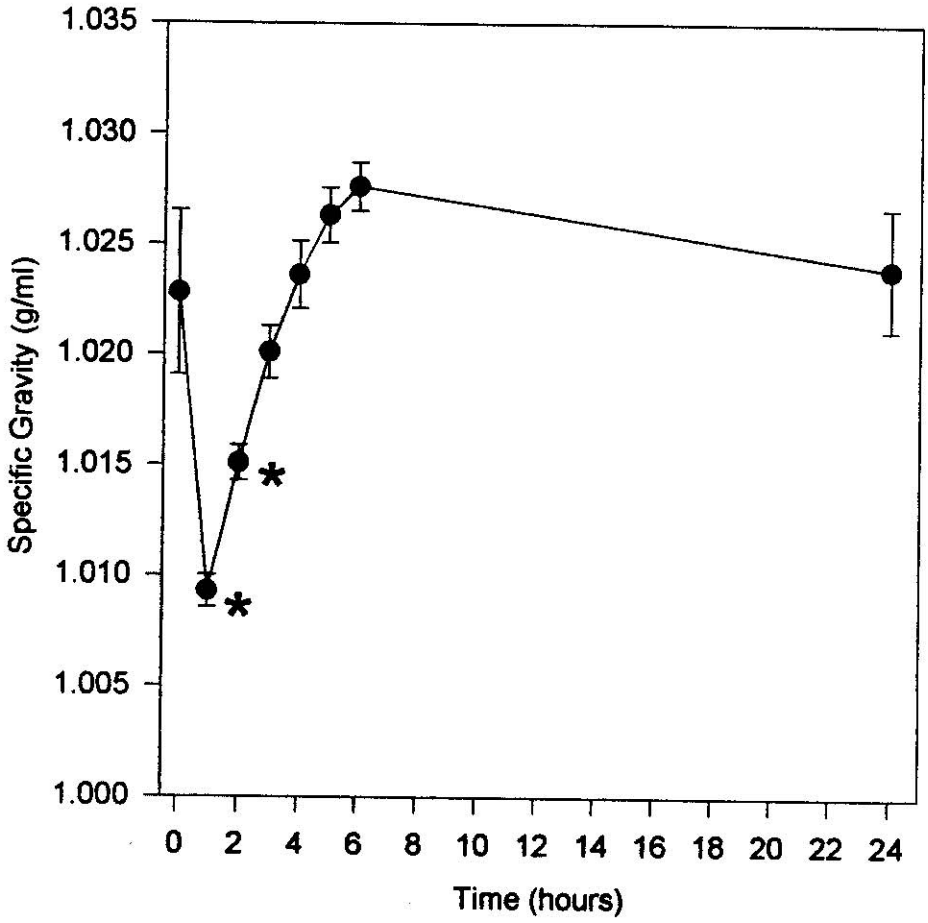

Figure 6. Mean specific gravity of urine samples (±SEM) following 250 mg intravenous administration of furosemide (n=10). *The asterisks indicate mean values are significantly different from control values.

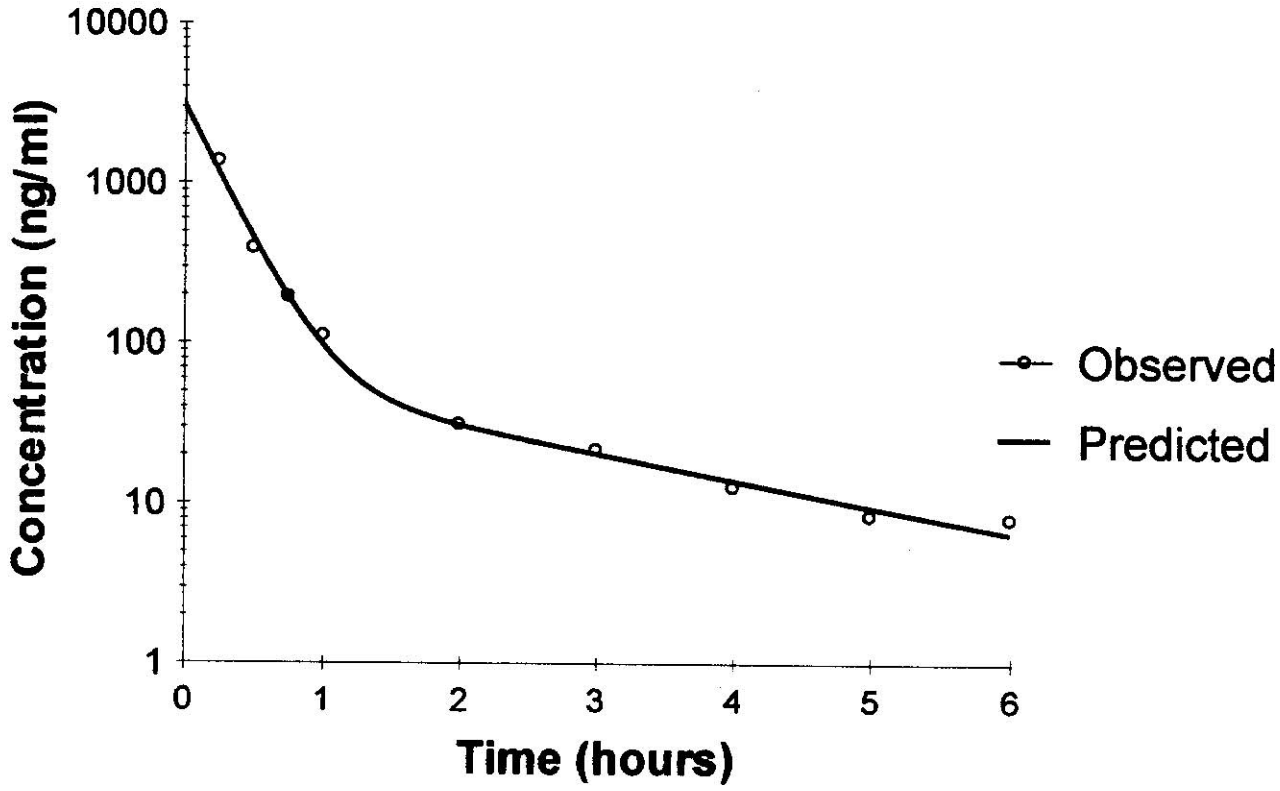

Figure 7. Mean observed vs. model predicted serum concentrations of furosemide following single intravenous administration of 250 mg furosemide (n=10).

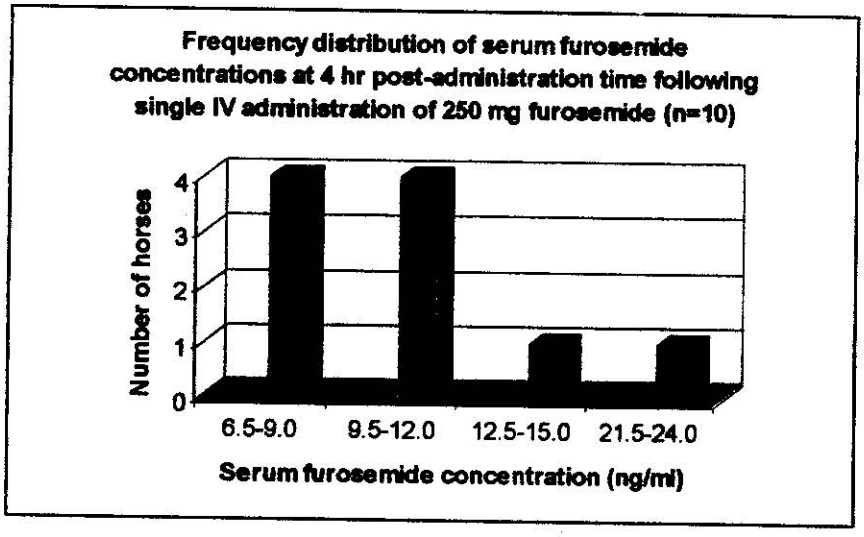

Figure 8. Frequency distribution of serum furosemide concentrations at 4 h post-time following IV administration of 250 mg furosemide (n=10) (Mean value=10.88 ng/ml).

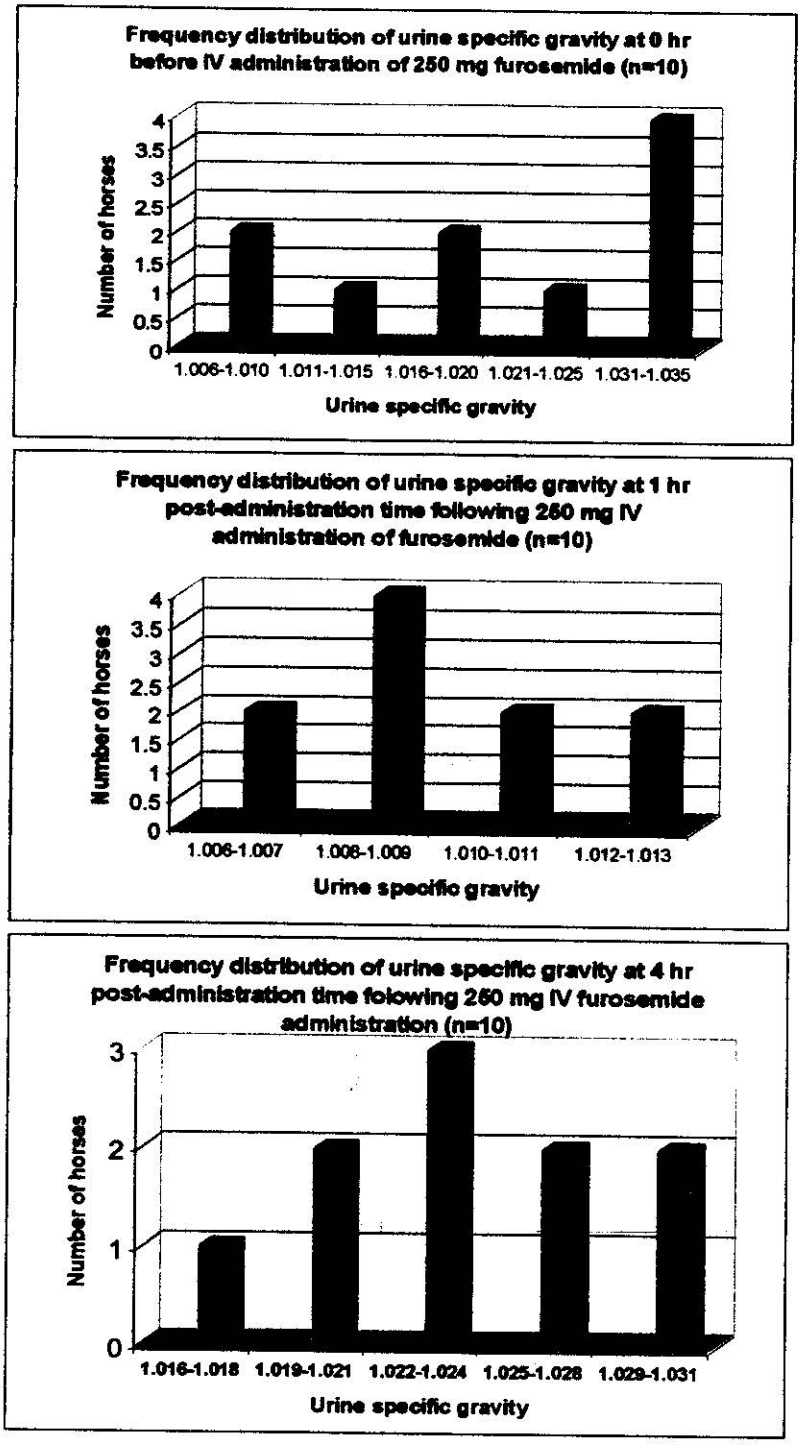

Figure 9. Frequency distribution of urine specific gravity before (0 h, mean value=1.023) and after (1 h, mean value=1.0093 and 4 h, mean value=1.024) IV administration of 250 mg of furosemide

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