

The Detection, Identification and Basic Pharmacology of Furosemide in the Horse

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In recent years, furosemide has become the diuretic of choice in the horse. It is thus the diuretic about which most is known in the equine, both clinically and analytically.¹ As such, it is essentially the only diuretic which is found in the urine of racing horses and my comments on the detection and pharmacology of diuretics in the horse will be limited to furosemide.

Characteristics of the Molecule

The detection of furosemide in equine plasma and urine depends on four properties of the furosemide molecule² outlined in Fig. 1. By virtue of its carboxyl (COOH) group, furosemide is an acidic drug and thus extracts from plasma or urine under acidic conditions. Because of its ring structures and sulfonamide substituent, furosemide readily fluoresces and can be detected on thin layer plates simply by shining long wave UV light on it.² By virtue of its chlorine atom, furosemide is electron capturing and can thus be detected by gas chromatography with electron capture detection. If the furosemide molecule is hydrolyzed under strongly acidic conditions, an NH₂ group is made available which yields a bright red color in the Bratton-Marshall reaction. Any or all of these properties may be used by the analyst to detect and/or confirm the presence of furosemide in equine plasma or urine.

Detection Procedures

A typical, if perhaps somewhat abbreviated drug detection scheme of the type that is used by race track testing laboratories³ is presented in Fig. 2. In this flow diagram, the possible pathways taken by furosemide are indicated by the heavy arrows. In this scheme, a sample of plasma or urine containing furosemide would be acidified with HCl, extracted into dichloromethane, and the extract concentrated by evaporation. This extract would then be subjected to thin layer chromatography (TLC) on silica gel plates in a chloroform, cyclohexane and acetic acid system.² After chromatography, the plates are inspected under long wave UV light for bright blue fluorescent spots at an R_f of about 0.15, corresponding with that of furosemide. Because furosemide is a permitted medication in Kentucky, the screening process usually stops at this point. However, for confirmation or unequivocal identification of the presence of furosemide for forensic purposes, further experiments would be carried out.

For further confirmation, the sample suspected of containing furosemide would be run again in the thin layer system with authentic furosemide running on each side of it. At the end of the "run" the plate(s) would again be examined under long wave UV light for the presence of fluorescent spot(s) corresponding with the authentic furosemide spot(s). To further confirm the

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CHARACTERISTICS OF FUROSEMIDE MOLECULE

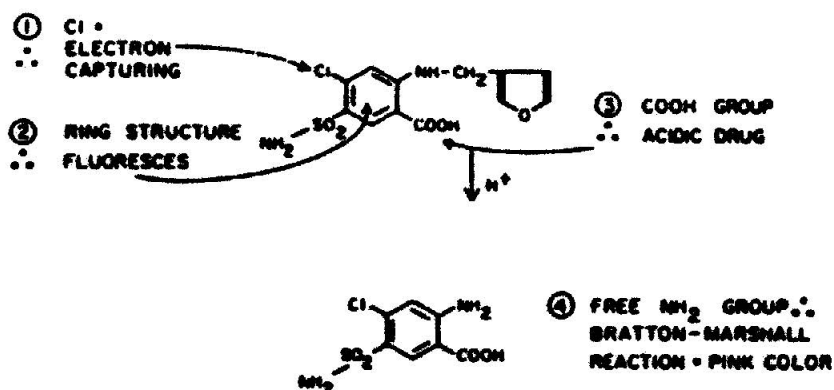


Fig. 1. Characteristics of the furosemide molecule useful in analytical work.

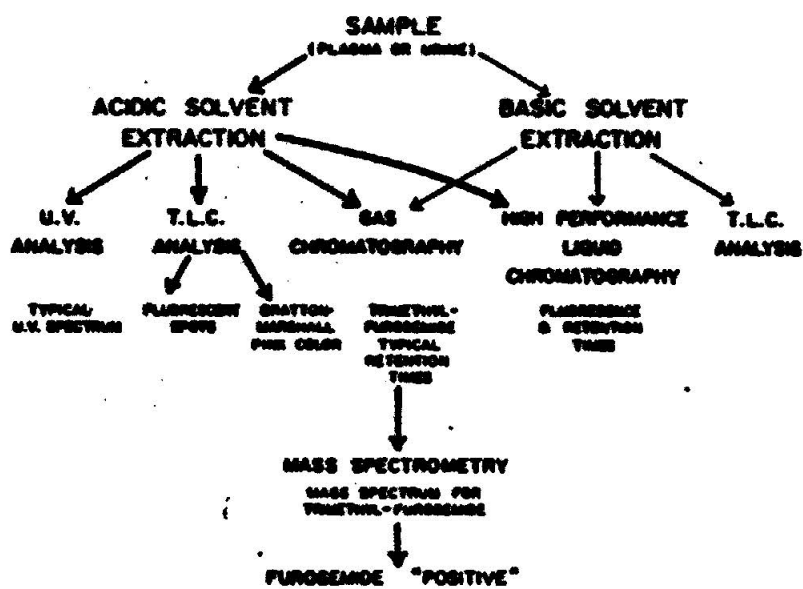


Fig. 2. Flow diagram for furosemide in a drug testing scheme.

presence of furosemide and obtain a permanent record of its presence, the plate can be sprayed in sequence with 5.0% H_2SO_4 , 0.7% NaNO_2 , 1.0% ammonium sulfamate, and 0.5% N(1-naphthyl)ethylene diamine dihydrochloride (N.E.D.D.). During these maneuvers, the H_2SO_4 hydrolyzes the furosemide to free the NH_2 group which then reacts with the Bratton-Marshall reagents. This sequence of sprays gives a bright pink spot corresponding to the position of furosemide on the control plates. This color spot (Fig. 3) is stable for months to years.²

Confirmation

While both of these TLC methods are useful for routine screening, unequivocal confirmation of the presence of furosemide in a sample requires gas chromatography and mass spectrometry. The difficulty with trying to "run" furosemide on a gas chromatograph is that the carboxyl and sulfonamide groups tend to make this molecule stick to GC columns and chromatograph poorly. Therefore, the approach is taken of reacting the furosemide with methyl-iodide to yield a derivative, trimethylfurosemide.² In this derivative, both the carboxyl and sulfonamide groups are methylated (Fig. 4) and this methylated derivative runs well on GC (Fig. 5). Using this method, furosemide may be readily identified and quantitated in equine plasma and urine samples.² Suspected furosemide may also be unequivocally identified for forensic work by using this methylation reaction on the sample in question.

The finding of gas chromatographic retention times similar to those of trimethylfurosemide and a mass spectrum indistinguishable from that of trimethylfurosemide in a forensic sample would constitute very good evidence that furosemide was present in the original sample.

Pharmacokinetics

Having developed a useful quantitative method for furosemide, we next investigated its kinetics in the horse. Figure 6 shows that after rapid intravenous (IV) injection of furosemide, plasma levels of the drug drop very rapidly, with an apparent half-life of about 35 minutes. Work in other species has shown that plasma levels of furosemide drop rapidly because the drug is actively pumped out of plasma into urine in the renal tubules (Fig. 7). This pumping accounts for both the rapid onset of action of Lasix and results in at least 60% of a dose of Lasix being excreted unchanged in the urine. This is a very high proportion of drug to be excreted unchanged in the horse and aids considerably in the detection of this drug in equine urine.²

Mode of Action

Once furosemide gets into the renal tubule, it acts to inhibit active reabsorption of chloride by the ascending limb of the loop of Henle.² Since sodium and water reabsorption normally passively follow chloride reabsorption, this blockade leads to chloruresis, naturesis and diuresis.⁴

Dose and Time Response Data

After IV injection, 0.4 mg/kg (about 4 ml) of furosemide produces four liters of urine within about 40 minutes (Fig. 8). A dose of 1 mg/kg (about 10 ml) produces about 8.5 liters of urine, mostly within one hour. A relatively

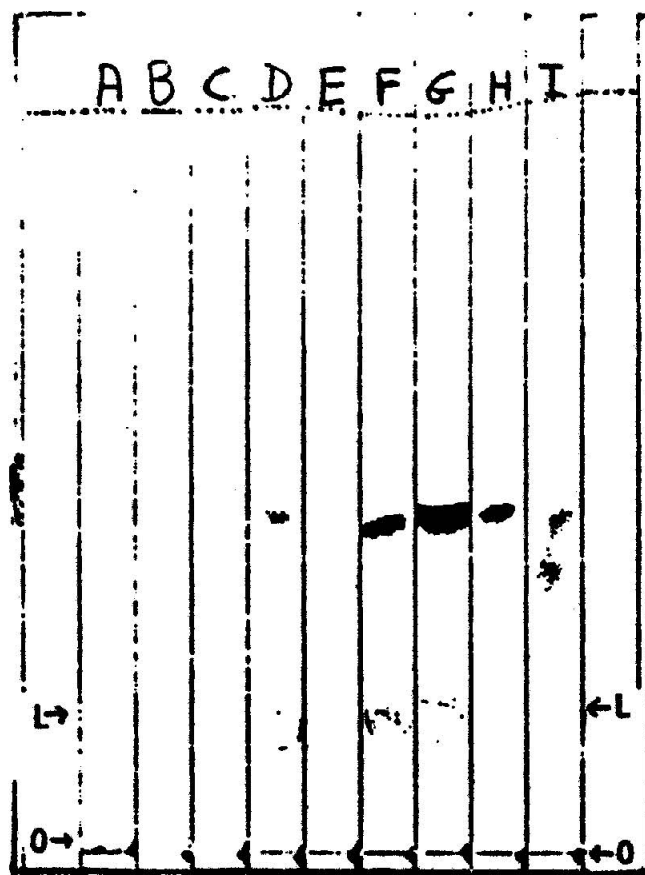


Fig. 3. Thin layer chromatogram of furuscicide recovered from equine urine.

A thoroughbred mare was dosed with 1 mg/kg furuscicide IM and urine samples taken over the next 24 hours. For detection of furuscicide, 14 ml of urine was acidified to a pH of 2.0 by the addition of about 10 drops of concentrated HCl. Four milliliters of dichloromethane (DCM) was then added to the system, the whole "vortexed" for about 5 minutes and then centrifuged. The urine layer was discarded and the DCM layer transferred to another tube and evaporated to dryness. The residue was then taken up in about 100 ml of DCM and streaked on silica gel 60, F254 plates. The plates were then developed in a solution of 6 parts chloroform, 4 parts cyclohexane, and 1.5 parts glacial acetic acid for about 90 minutes. After developing, the plates were air-dried and immediately examined under long wave UV light for bright spots corresponding with furuscicide. This is the usual method used for routine screening in Kentucky. To develop a permanent record these plates were then sprayed in sequence with 50% H₂SO₄, 0.7% NaNO₂, 1.0% ammonium sulfamate, and 0.5% N (1-naphthyl) ethylene diamine dihydrochloride (NEDD). A bright pink spot developed immediately corresponding to the position of furuscicide on the control plates. This color spot was stable for several weeks. The chromatograms are as follows: A, control urine prior to furuscicide; B, urine spiked with 15 µg furuscicide; C, D, E, F, G, H, urine samples, 2, 4, 8, 12, and 24 hours post dosing; I, urine spiked with 5 µg/ml furuscicide. The arrows marked L point to the furuscicide spots, which are bright red in color. The arrows marked O point to the origin. The circles on samples B and H encompass a faint pink spot in B, and the position of a trace of fluorescence in H. Sample I yielded no detectable fluorescence or color. This thin layer plate is typical of about 12 experiments.

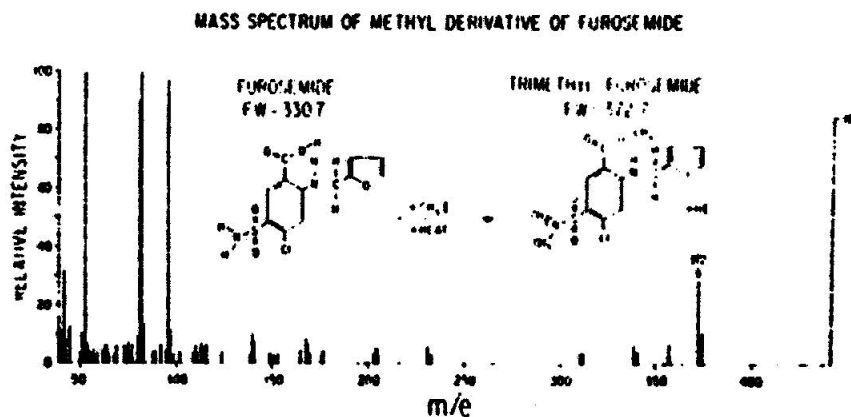


Fig. 4 Methylation of Furosemide and Mass Spectrum of Trimethylfurosemide.

To methylate furosemide, 20. ml of 0.2 M NaOH, 50 μ l of THAHN (tetrahexylammonium-hydrogen-sulfate), and 5.0 ml of 0.5 M methyl iodide (in DCM) were added to each tube. The tubes were capped tightly and secured horizontally in a shaker/water bath at 50°C for 20 minutes. Methylation occurred rapidly under these conditions (Fig. 2). The samples were then removed from the water bath, centrifuged, and stored at 4°C. Allowing the tubes to stand for a period of time at this point gave cleaner chromatograms. The aqueous phase was removed and the dichloromethane (DCM) phase decanted into another tube and evaporated to dryness at 60°C in a water bath. Two milliliters of hexane and 1.0 ml of 0.2 M NaOH were added to each tube, "rotorocked," and centrifuged. Samples were then ready for gas chromatographic analysis or mass spectrometry.

large dose of furosemide, 4 mg/kg (about 40 ml) yields about 21 liters of urine within about two hours. These are all relatively rapid responses and at the lower doses, the diuretic effect is limited by rapid excretion of the drug. At higher dose levels or after repeated dosing, the pharmacological effect is more likely limited by the availability of extracellular fluid, and under these conditions, the animal becomes relatively resistant to the effects of furosemide.⁷

One way to prolong the pharmacological effects of furosemide in the horse is to give the drug intramuscularly (IM). When given in this way, plasma levels of the drug peak within minutes, but then decline at only about one-third the rate that is observed after IV injection of the drug. In this way, intramuscular administration of the drug prolongs the plasma half-life of the drug and thus, its pharmacological effects. The upshot is that about 50% more water and sodium is lost after IM injection of the drug than after IV injection.⁷

Relationship Between Plasma Levels of Drug and Diuretic Effect

If the horse is well hydrated, the diuretic response to furosemide is very closely related to its plasma levels. Figure 9 shows the decline in plasma levels of furosemide after its IV and IM injections matched with the rate of decline of the diuretic response after each dose. The data show that in each case, the rate of decay of the diuretic response was just a little faster than the rate of decline of plasma levels of the drug and both can almost be superimposed.

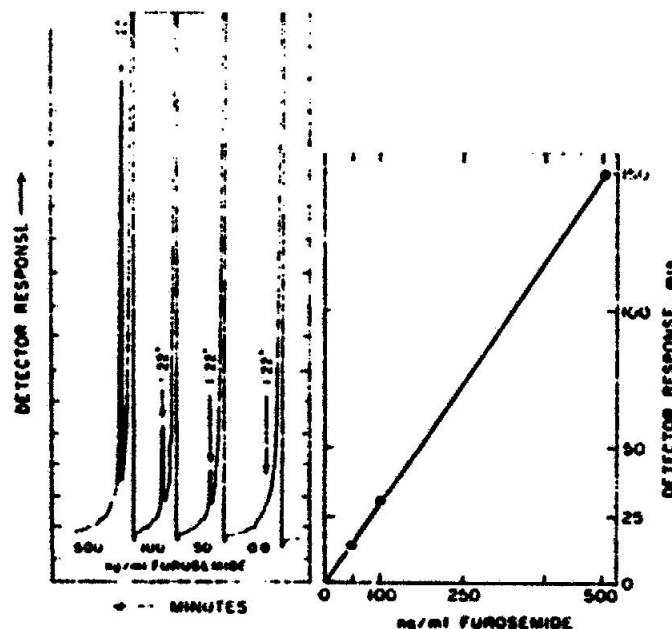


Fig. 5. Gas Chromatograms of Trimethylfurosemide.

The left hand panel shows gas chromatograms of deuterized "spiked" furosemide standards. The right hand panel shows that detector response was linearly related to the amount of furosemide added over the range of 50-500 ng/ml furosemide. A Perkin-Elmer 3920-B gas chromatograph equipped with a ^{63}Ni electron capture detector was used for all determinations. This machine was equipped with a 6 foot x $\frac{1}{8}$ inch O.D. glass column packed with 3% OV-101 absorbed on 100/200-mesh Gas Chrom Q. The injection port temperature was 310°C , column temperature 200°C , interface 300°C , and ECD 300°C . Purified N_2 was the carrier gas at a flow rate of 55 ml/minute. A sample volume of 2.0 μl iters was used throughout.

The data suggest that the diuretic response to furosemide is directly related to its plasma levels.

Effect on Cation Levels in Plasma and Urine

The effects of increasing doses of furosemide on urinary output and cation concentrations are shown in Fig. 10. While increasing doses of furosemide produce a graded increase in urinary volume, they produce what is essentially an all or none increase in urinary Na^+ and a graded decrease in urinary K^+ . The net result of this is that increasing doses of furosemide produce increasing Na^+ and H_2O losses, but the total loss of K^+ does not tend to increase as the dose of furosemide is increased.

Despite the fact that the net effect of furosemide on K^+ excretion is small, serum (K^+) is quite sensitive to furosemide. As shown in Fig. 11, serum levels of K^+ drop rapidly after a dose of furosemide, to 20% below normal after a single dose and up to 30% below normal after repeated doses.⁷ After a single dose, however, plasma levels of K^+ , total solids, and packed cell volumes rapidly return to control values.⁷

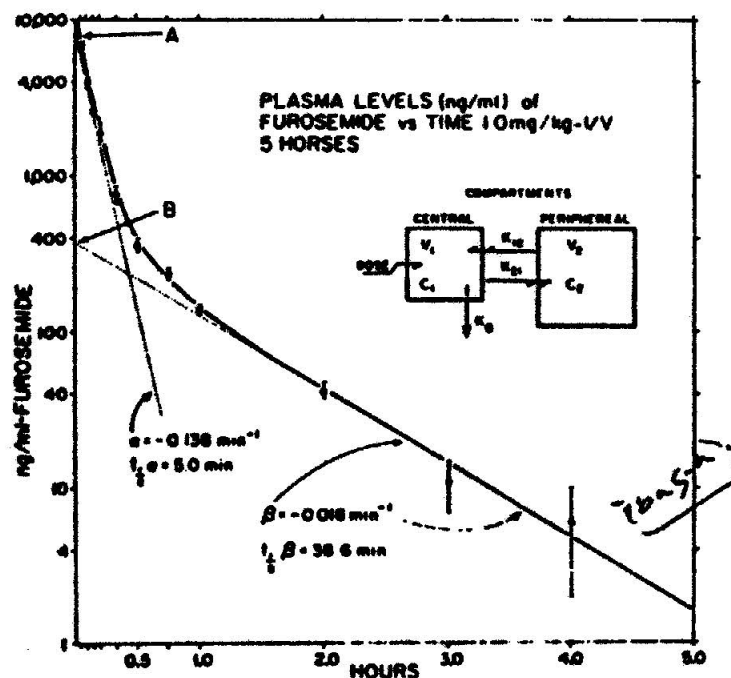


Fig. 6. Plasma levels of Furosemide after 2 mg/kg IV.

Plasma levels of furosemide after its rapid IV injection. The solid circles (●) show plasma levels of furosemide after rapid IV administration of 1 mg/kg furosemide. The solid line from 1.5 hours on is that of a least squares regression fit to all points after 45 minutes. The rest of the solid line was fitted by eye to the data points. The dotted lines A and B represent the 2 components into which this curve may be resolved, the Alpha or distribution phase with a half-life of about 5 minutes, and the Beta or elimination phase with a half-life of about 39 minutes. The inset shows a 2-compartment open model which may be used to describe these data.

Effects on Detection of Other Drugs

Treatment with furosemide has not reduced the plasma levels of any drug studied to date. In addition, furosemide has no forensically significant effects on urinary concentrations of procaine or methylphenidate. Since these are both basic, relatively lipid soluble drugs, a reasonable conclusion from these studies might be that the urinary concentrations of basic, lipid soluble drugs are not likely to be affected by furosemide.⁶

On the other hand, furosemide reduces urinary concentrations of phenylbutazone (Fig. 12) and the major glucuronide metabolite of pentazocine (Fig. 13) up to 50-fold. These experiments suggest that the urinary concentration of acidic drugs such as the nonsteroidal anti-inflammatory agents and of water soluble drug metabolites are likely to be reduced by furosemide treatment. These actions of furosemide are likely to significantly interfere with routine forensic screening for drugs in urine.⁶

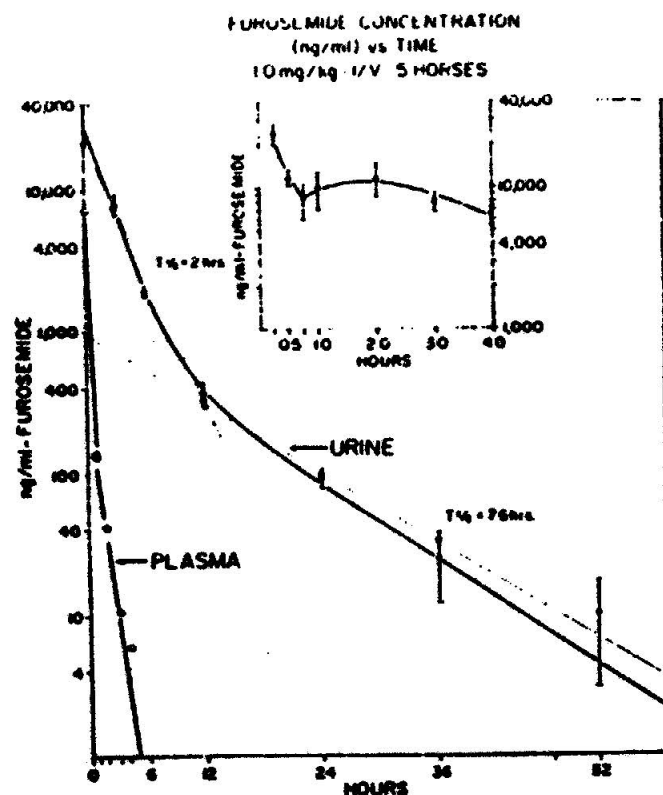


Fig. 7. Urinary Concentrations of Furosemide after 1 mg/kg IV.

The solid circles (● - ●) show urinary concentrations of furosemide after IV administration of 1 mg/kg of furosemide. The inset shows a detail of urinary concentration during the first 4 hours post dosing. The dotted lines represent least squares fit to urinary concentrations of the drug observed over the first 12 hours and for subsequent 40-hour periods together with the respective apparent half-lives. The open circles (○ - ○) show the corresponding plasma levels (Fig. 6) replotted for comparative purposes. All data points are means \pm SEM of experiments on 5 horses.

Performance Effects

Time trials on standardbred horses at the University of Kentucky and The Ohio State University have not shown any significant differences between the times to pace one mile of furosemide-treated and control horses. In an extension of these studies, we analyzed race times pre- and post-furosemide for 58 standardbred horses running at the Louisville Downs meet last summer (1977) (Table 1). The results of this analysis show no significant difference between the times of these horses before and after they went on furosemide. The data strongly suggest that pre-treatment with Lasix does not change the time to pace one mile for standardbred horses.⁷

TABLE I
Effect of Medication with Furosemide on the Performance of
Horses Racing at Louisville Downs, Summer, 1977

	# of Horses	# of Trials	Mean Times	S.E.M.
Pre-Furosemide	58	160	128.5925	0.2031 $t < 0.00$
With Furosemide	58	232	128.7366	0.1594 (t for significance should be > 3.0)

At this meet furosemide was the only permitted medication, and its use was monitored by urinalysis. Horses could elect to go on furosemide at any time throughout the meet, but once on furosemide had to stay on it. Performance times for horses pre- and post-furosemide treatment were obtained from the meet programs and compared. Only times on good or fast tracks were taken. For the 58 horses selected, 160 prefurosemide times were available and 232 post-furosemide times. A randomized block design was used where each horse represented a block. After adjusting for blocks (i.e., differences between horses), there was no significant difference between treatments (i.e., times on and off furosemide).

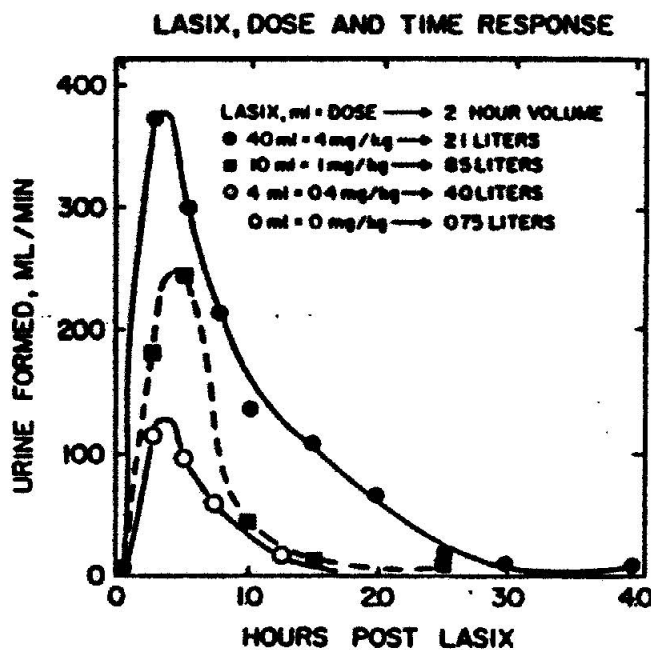


Fig. 8. Urinary Volume Response to Increasing Doses of Furosemide.

The solid circles (●) show the diuretic response to 4 mg/kg (40 ml) of furosemide IV at zero time. The squares (■) and open circles (○) show the response to 1 mg/kg (10 ml) and 0.4 mg/kg (4 ml), respectively. All urine samples were obtained by bladder catheterization and the symbols represent the mean number of ml/minute excreted in the preceding time period. All points are the means of at least four experimental determinations.

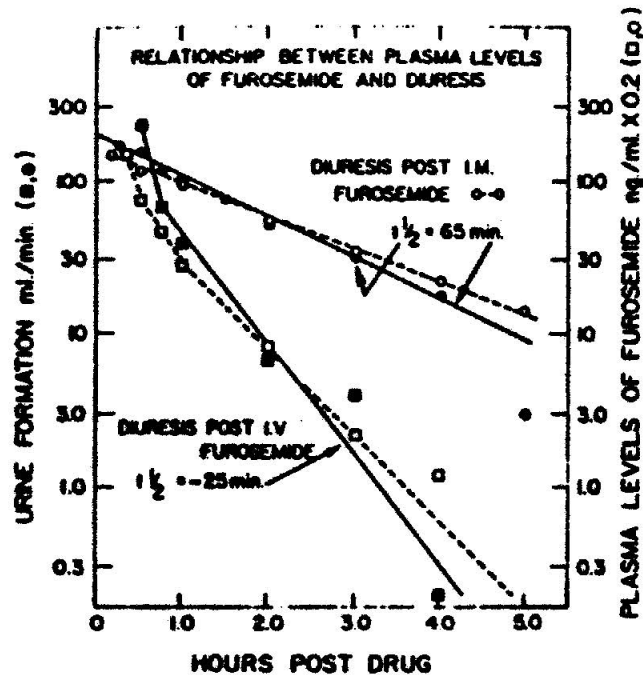


Fig. 9. Relationship between plasma levels of furosemide and diuresis.

The solid symbols and lines show rates of urine formation in ml/minute after IV injection (solid squares, ■-■) and after IM injection (solid circles, ●-●) of 1 mg/kg furosemide. Control rates of urine formation were subtracted from all values so the points represent diuresis due to furosemide only. The open squares (□-□) and circles (○-○) show plasma levels of drug after similar doses of furosemide. Plasma levels of furosemide were superimposed in urinary flow rates by multiplying all plasma levels by 0.2. The approximate half-lives for the diuretic effect after each route of administration compare with kinetically determined plasma half-lives for furosemide of about 30 and 86 minutes, respectively.

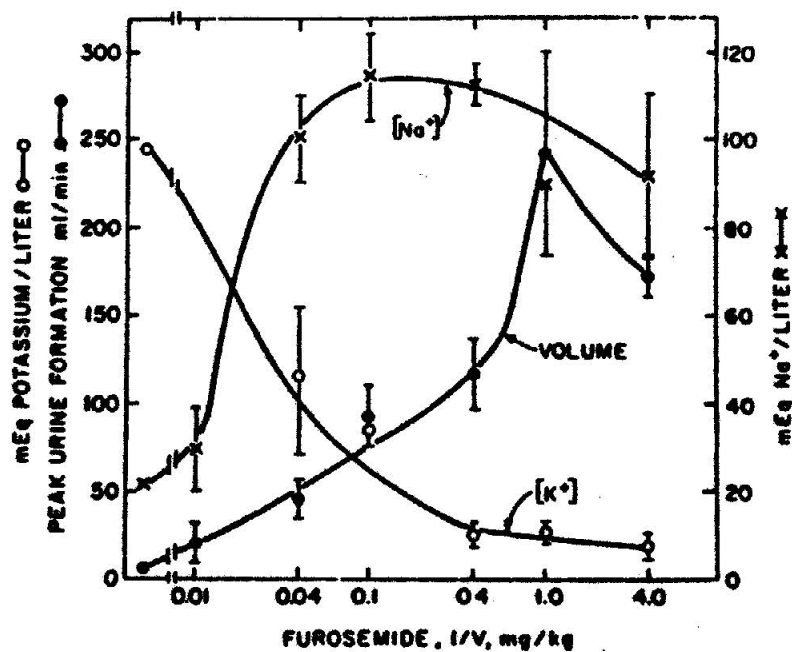


Fig. 10. Dose response relationships of urinary output and cation concentrations at peak diuresis due to intravenous furosemide.

Furosemide at the indicated doses was given by rapid intravenous injection to horses. The solid circles (● - ●) show peak urinary output at each of the indicated doses. The crosses (x - x) and open circles (○ - ○) show urinary concentrations of Na⁺ and K⁺, respectively, at the indicated dosage levels. All experiments except those at 0.01 mg/kg (2 horses) are the means of experiments on 4 different horses ± standard deviation.

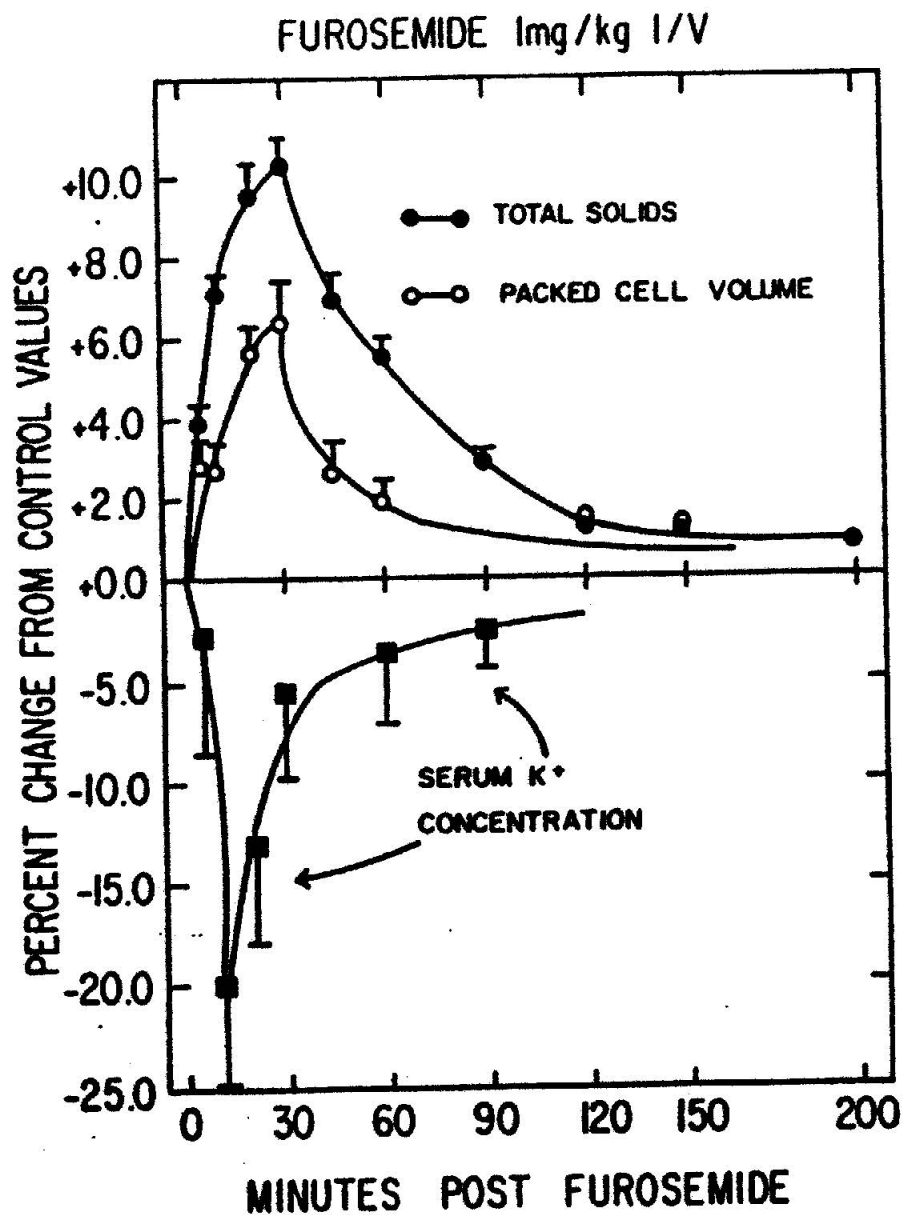


Fig. 11. Effect of 1 mg/kg furosemide intravenously on blood values.

Horses were administered 1 mg/kg furosemide intravenously and blood samples drawn at the indicated times. The solid circles (●-●) show the percent increase in total plasma solids, while the open circles (○-○) show the percent increase in packed cell volume following furosemide. The solid squares (■-■) show the percent decrease in serum K⁺. Control serum K⁺ levels were 3.76 ± 0.13 mEq/liter. All data are expressed as percent change from predrug controls and are the mean ± standard error of the mean of experiments on 6 horses.

EFFECT OF FUROSEMIDE TREATMENT ON URINARY PHENYLBUTAZONE CONCENTRATION

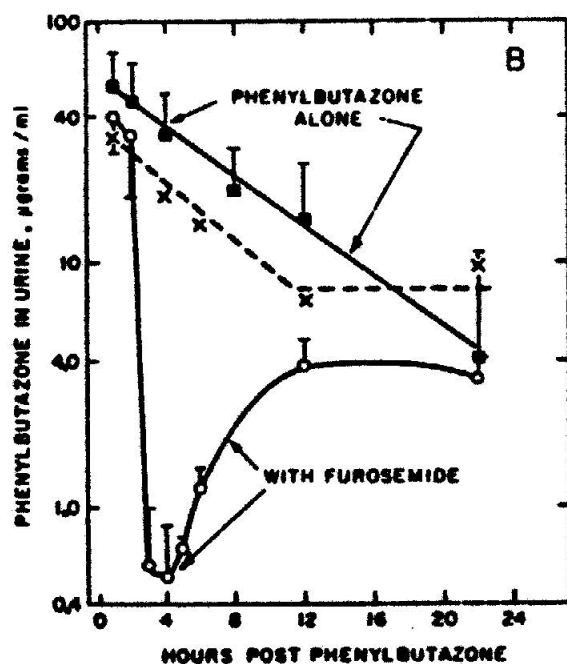


Fig. 12. Effect of furosemide on urinary concentrations of phenylbutazone.

The crosses (x - x) and solid squares (■ - ■) show urinary concentrations of phenylbutazone in 4 horses administered 6.6 mg/kg IV at zero time. The open circles (○ - ○) show urinary concentrations of phenylbutazone in 4 horses in which the phenylbutazone was followed 2.5 hours later by 1 mg/kg of furosemide IV.

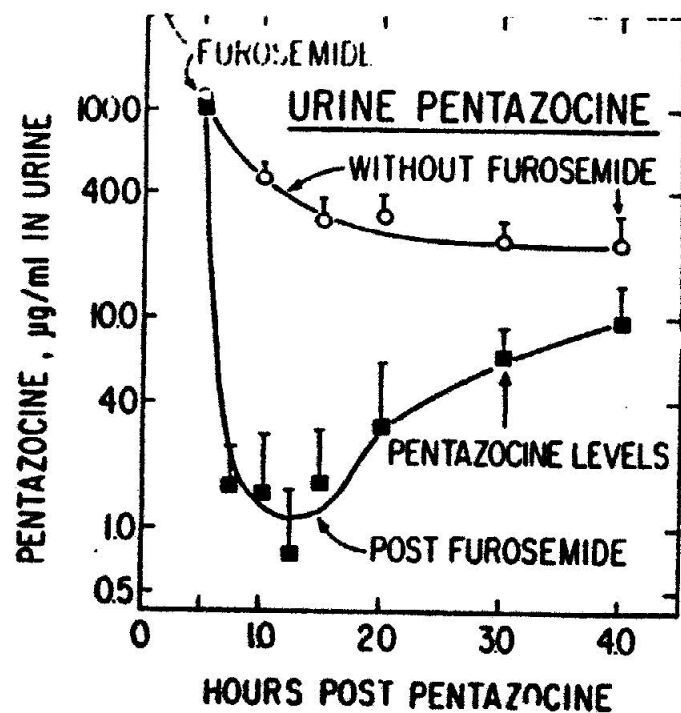


Fig. 13. Effect of furosemide on urinary concentrations of a glucuronide metabolite of pentazocine.

Horses were injected IV with 0.33 mg/kg pentazocine at indicated zero time. The open circles (1-4) show urinary concentrations of a glucuronide metabolite of pentazocine in control horses. The solid squares (5-8) show urinary concentrations of this metabolite in horses treated with 1 mg/kg of furosemide IV 30 minutes postpentazocine. All data points are mean \pm standard error of mean of experiments on at least 4 different horses.

Summary

1. Furosemide can be detected in equine plasma and urine by colorimetric, T.L.C. or gas chromatographic methods.
2. Methylation to trimethylofurosemide and gas chromatographic analysis is the most satisfactory quantitative method. Mass Spectrometric analysis of this derivative may be used for unequivocal identification of furosemide.
3. Administered intravenously, Lasix[®] is a short acting drug, the bulk of which is excreted unchanged in the urine.
4. Lasix[®] transiently increases total plasma solids and hematocrit and decreases plasma K^+ and pulmonary blood pressure.
5. Intramuscular injection of Lasix[®] prolongs plasma levels of the drug and its diuretic response.
6. Lasix[®] can reduce urinary concentrations of phenylbutazone and the major metabolite of pentazocine up to 50-fold.
7. Lasix[®] does not affect the times to pace one mile of Standardbred horses.

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