

Immunoassay Detection of Drugs in Racing Horses: Detection of Ethacrynic Acid and Bumetanide in Equine Urine by ELISA*

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Abstract

We have raised antibodies and developed one-step enzyme-linked immunosorbent assays (ELISA) for the diuretics ethacrynic acid and bumetanide as part of a panel of pre- and post-race tests for high potency drugs in racing horses. These ELISA tests are rapid (completed within one hour), sensitive, and can be read by eye. The ELISA detects ethacrynic acid at a drug concentration for half-maximal inhibition (i-50) of about 2.5 ng/mL for the parent drug. After dosing horses intravenously with 5 mg ethacrynic acid per horse, the parent drug or its metabolites are detectable in urine for at least 8 hours. The bumetanide ELISA has an i-50 for the parent drug of about 2.0 ng/mL and will detect bumetanide or its metabolites for about 8 hours in urine after intravenous administration of a 1.7-mg dose per horse. Both antibodies are relatively specific for each drug and do not cross-react with other commonly used diuretics or other acidic compounds often found in post-race equine urine samples. Ethacrynic acid and bumetanide are potent diuretics suspected of being illegally substituted for furosemide in certain racing jurisdictions. Development of these rapid, sensitive, and simple tests for these agents will allow more effective pre- and post-race control of the use of these agents in racing horses. Both tests have recently uncovered several "positives" for these medications in a midwestern racing jurisdiction.

Introduction

Ethacrynic acid ([2,3-dichloro-4-(2-methylene-1-oxobutyl) phenoxy] acetic acid, Sodium Edecrin®) is a potent diuretic acting on the ascending loop of Henle, as well as the proximal and distal tubules, in order to block the reabsorption of filtered sodium. Ethacrynic acid is widely used in human medicine for the treatment of edema associated with congestive heart failure, cirrhosis of the liver, and renal disease (1).

Bumetanide [(3-aminosulfonyl)-5-(butylamino)-4-phenoxy-

benzoic acid, Bumex®] is structurally related to the commonly used diuretic furosemide. Like ethacrynic acid, it is a loop diuretic and exhibits even greater diuretic activity, with a potency on the order of 50 times that of furosemide (2).

Both drugs are commonly used in human medicine. Ethacrynic acid and bumetanide have been detected in blood and urine by thin-layer chromatography (TLC) (3), radioimmunoassay (RIA) (4), gas chromatography (GC) (5,6), and liquid chromatography (LC) (7,8). Reported detection limits range from 1 to 50 ng/mL.

These drugs are also suspected of being used illegally in racing horses for the treatment of exercise-induced pulmonary hemorrhage (EIPH), especially in racing jurisdictions where the use of furosemide is prohibited. An additional concern over the use of diuretics is the possibility that these drugs will interfere with the detection of other illegal medications by diluting drugs or drug metabolites in equine urine (9). Therefore, we have developed enzyme-linked immunosorbent assays (ELISA) for ethacrynic acid and bumetanide as part of a panel of ELISAs for drugs in racing horses.

Materials and Methods

Horses. For the in vivo studies, Thoroughbred or Standardbred mares (400–450 kg) were administered ethacrynic acid or bumetanide. These horses were maintained at pasture until approximately 24 hours before dosing, at which time they were placed in box stalls (17 m²) and allowed free access to hay and water. All control samples were obtained on the day of the dosings.

Dosing and sampling. Injectable and chemically pure ethacrynic acid (Sodium Edecrin; Merck Sharp & Dohme, West Point, PA) and bumetanide (Bumex; Hoffman-LaRoche, Nutley, NJ) were obtained for use in assay development and test evaluation. All drugs were administered by rapid intravenous (iv) bolus injection into the jugular vein. All urine samples were collected by bladder catheterization and stored frozen until assayed.

ELISA method. The ELISAs developed here are similar to the ELISA formats described in previously published reports by Stanley et al. (10) and by Tobin et al. (11). The anti-ethacrynic acid antibody or anti-bumetanide antibody was coated onto flat-

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bottom microtiter wells (Costar Inc., Cambridge, MA) as described by Voller et al. (12). Ethacrynic acid and bumetanide were linked by the method described by Rowley et al. (13) to horseradish peroxidase (HRP) to give rise to a covalently linked drug-HRP complex. Urine samples were diluted as necessary with assay buffer (0.1M phosphate buffered saline, pH 7.6, with 1% bovine serum albumin) prior to testing. All assays were performed at room temperature. The assay was started by adding 20 μ L of the standard, test, or control samples to each well, along with 180 μ L of the drug-HRP solution. During the test, the presence of drug in the sample competitively prevented the binding of the drug-HRP complex to the antibody. After incubation the wells were washed, and 150 μ L of TMB Microwell Peroxidase substrate (Kirkegaard and Perry, Gaithersburg, MD) was added. Because the HRP enzyme is responsible for the color-producing reaction in the ELISA, the concentration of drug in the sample is inversely related to the optical density of the test well. The optical density (OD_{650}) of each test well was determined at 650 nm with an automated microplate reader (Bio-Tek Inc., Winoski, VT) after a period of color development, usually approximately 60 minutes after addition of substrate.

Mass spectral confirmation of ethacrynic acid. The presence of ethacrynic acid in post-race urine samples screened by the ethacrynic acid ELISA was confirmed by gas chromatography/mass spectrometry (GC/MS) for up to 6–8 hours after a dose of 5 mg per horse. To a screw-top glass culture tube were added 10 mL of the urine sample, 2 mL of saturated aqueous KH_2PO_4 , and 0.5 mL of concentrated HCl. The mixture was extracted with 6 mL of dichloromethane by rotation for 15 min. The aqueous layer was removed, and the organic layer was washed with 5 mL of 5% aqueous lead acetate. The washed dichloromethane extract was evaporated to dryness under a stream of dry nitrogen. The extract was dissolved in 30 μ L methanol. The samples were analyzed on an HP 5890 GC (Hewlett-Packard, Palo Alto, CA) equipped with an HP-1 methylsilicone capillary column and a model 5970 mass selective detector. Ethacrynic acid was detected as its methyl ester via methylation of 2 μ L of sample with trimethylanilinium hydrochloride in methanol, injected on the column.

Mass spectral confirmation of bumetanide. Bumetanide from a post-race equine urine sample was screened by the bumetanide ELISA and was isolated by solid-phase extraction (SPE) using Bond Elut Certify SPE cartridges (Varian Sample Preparation Products, Harbor City, CA) and an acidic-neutral extraction procedure. Briefly, the SPE cartridge was loaded with 5 mL of urine plus 2 mL of 0.1M potassium phosphate buffer, pH 6, rinsed with 1 mL of the phosphate buffer-methanol (80:20), then with 1 mL of 1M acetic acid, and followed by 1 mL of hexane. The bumetanide was eluted with 4 mL of methylene chloride. The resulting so-

lution was evaporated to dryness and reconstituted in 20 μ L of ethyl acetate. GC/MS analysis was performed on a Varian GC (Varian Instruments, Sunnyvale, CA) coupled to a Finnigan Inco 50 mass spectrometer (Finnigan MAT, San Jose, CA).

Results

The ability of added ethacrynic acid and potentially cross-reactive compounds to inhibit the ELISA reaction is shown in Figure 1. Increasing concentrations of ethacrynic acid inhibited the reaction, with half-maximal inhibition (I-50) occurring at about 2.5 ng/mL. The anti-ethacrynic acid antibody was tested for cross-reactivity with a group of diuretics and other acidic com-

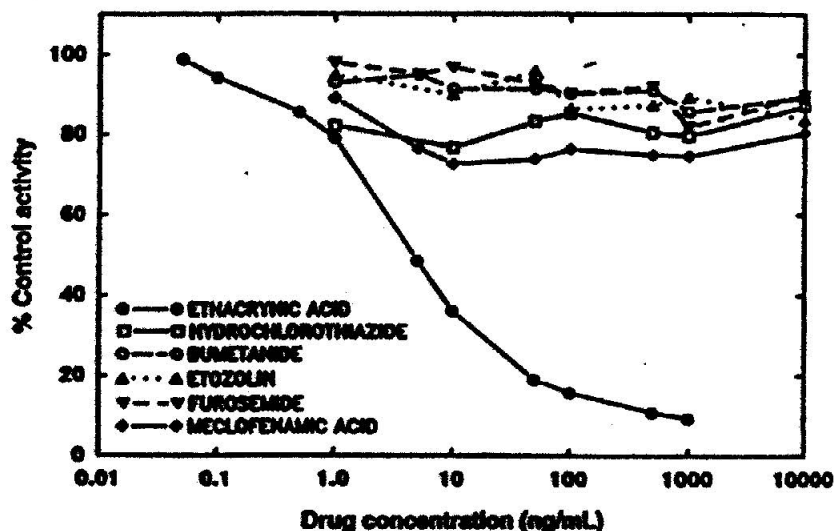


Figure 1. Sensitivity and cross-reactivity of the ethacrynic acid ELISA. Half-maximal inhibition (I-50) of the ELISA occurs at around 2.5 ng/mL ethacrynic acid, with no significant cross-reactivity associated with the other drugs tested.

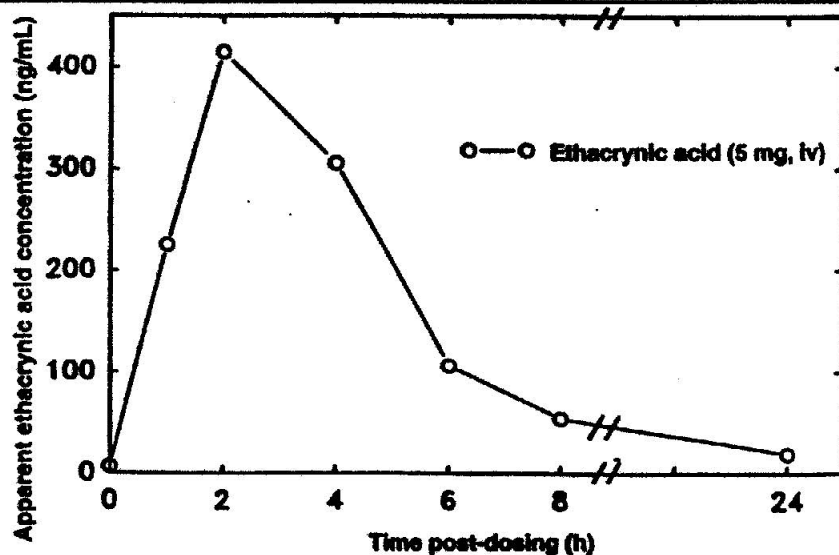


Figure 2. ELISA detection of ethacrynic acid in equine urine. The open circles (O—O) show the calculated apparent ethacrynic acid concentration in urine samples collected after intravenous administration of 5 mg of ethacrynic acid per horse.

pounds commonly found in post-race equine urine samples. Neither bumetanide, hydrochlorothiazide, furosemide, etozolin, nor meclofenamic acid produced any significant inhibition of the ethacrynic acid ELISA.

The ability of the ethacrynic acid ELISA to detect the presence of the drug following an iv dose of 5 mg per horse (0.012 mg/kg) was evaluated. Figures 2 and 3 show inhibition of the ELISA reaction by equine urine samples collected after treatment with 5 mg of ethacrynic acid intravenously. Figure 2 shows the dose-response curve expressed as apparent drug concentration, whereas Figure 3 shows the inhibition of the ELISA activity by the dosed-horse urine samples. The test easily detected ethacrynic acid in urine for up to 8 hours postdose, with significant inhibition of the ELISA color-producing reaction for up to 24 hours

after an intravenous dose of 50 mg per horse.

Unidentified substances present in many equine urine samples are known to partially inhibit the antibody-drug interaction of some ELISAs, causing a "background", or reduction of color (OD_{550}), in the assay. This is illustrated by the 56% inhibition of the urine sample collected predose from the horse dosed with ethacrynic acid (Figure 3). To determine the background interference in the ethacrynic acid ELISA, a set of 40 post-race urine samples, assayed by TLC as negative for ethacrynic acid by a state racing laboratory and furnished to us by that authority, was screened for apparent ethacrynic acid content using this ELISA. In order to reduce the interference of the background inhibition with the detection of the drug when using the ELISA, the 40 samples were diluted to the degree that none of the apparent drug concentrations exceeded the I-50 for that ELISA. As shown in Figure 4, the background concentrations of apparent ethacrynic acid equivalents seen in 40 equine urine samples diluted 1:4 with assay buffer were less than the I-50 value for ethacrynic acid in this assay. For comparison, the apparent ethacrynic acid concentration of a urine sample, diluted 1:4, collected from a horse 1 h after a 5-mg iv dose of ethacrynic acid is indicated by the arrow.

A similar series of experiments was performed for the evaluation of the bumetanide ELISA. The ELISA response to increasing concentrations of bumetanide and other diuretics and other commonly used acidic drugs is shown in Figure 5. Half-maximal inhibition of the ELISA by bumetanide occurs at about 2 ng bumetanide/mL. The anti-bumetanide antibody showed no cross-reactivity with ethacrynic acid, furosemide, flunixin, ibuprofen, meclofenamic acid, naproxen, phenylbutazone, or etozolin. There was insignificant cross-reactivity with the structurally similar compound *m*-aminobenzoic acid.

The bumetanide ELISA is sufficiently sensitive to detect bumetanide or its metabolites in equine urine for 8 hours following a 1.7 mg (4 μ g/kg) iv dose (Figures 6 and 7). In samples taken shortly after administration, the ELISA reaction was essentially completely inhibited. By 24 hours after administration the ELISA response had returned to about half the control value.

Forty post-race urine samples were tested by the bumetanide ELISA and the background distribution plotted (Figure 8). As with the ethacrynic acid ELISA, the samples required a 1:4 dilution to reduce the highest background apparent bumetanide concentration below the I-50 of the assay. For comparison, the apparent bumetanide concentration for a urine sample diluted 1:4, collected 1 h after an intravenous 1.7-mg dose, is indicated by the arrow.

When these tests were introduced into routine post-race testing in a midwestern state, they rapidly identified certain samples as "ELISA-positive" for ethacrynic acid and

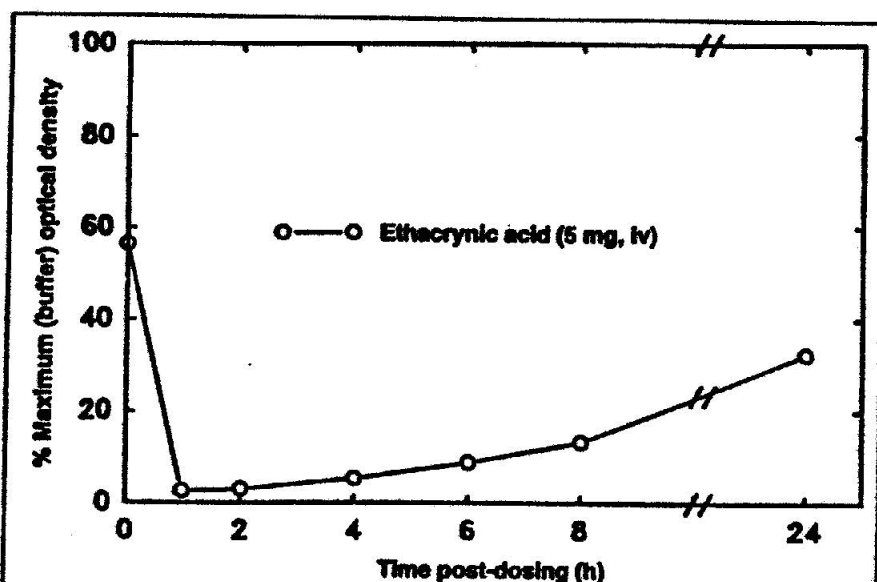


Figure 3. ELISA inhibition by ethacrynic acid dosed-horse urine samples. The open circles (○-○) show the decrease in OD_{550} in urine samples collected after intravenous administration of 5 mg ethacrynic acid per horse.

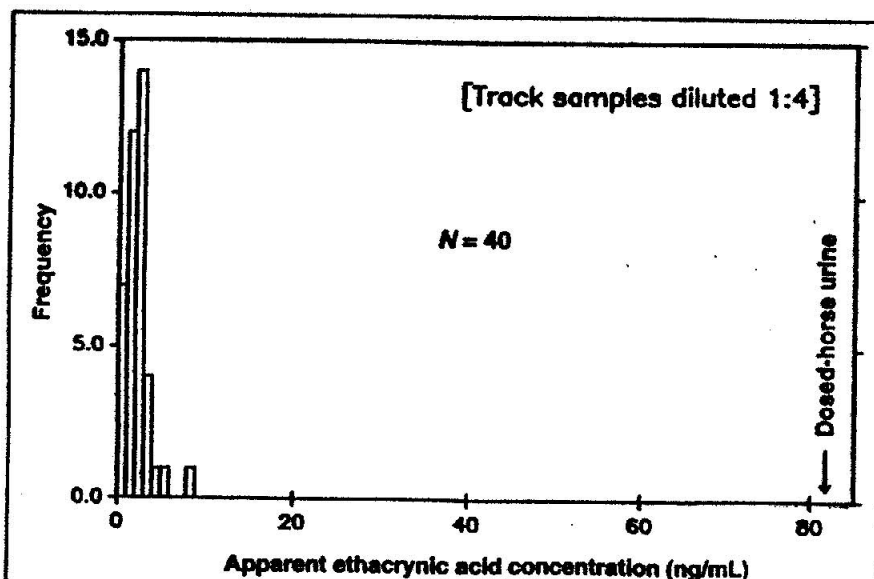


Figure 4. The frequency distribution of apparent ethacrynic acid concentrations for 40 ethacrynic acid negative post-race urine samples. The apparent ethacrynic acid concentration of a sample collected 1 h postdose from a horse administered 5 mg ethacrynic acid intravenously is indicated by the arrow. All samples were diluted 1:4 with assay buffer.

bumetanide. When these samples were analyzed by GC/MS to confirm or deny the presence of these drugs, typical spectra for ethacrynic acid methyl ester and tetramethyl bumetanide (Figures 9 and 10) were obtained. Because full mass spectra are considered chemically definitive evidence for the presence of these drugs, these samples, along with others on which equivalent data were obtained, were reported as regulatory "positives" for these drugs.

Discussion

Ethacrynic acid and bumetanide are high potency diuretics that have essentially the same pharmacological effects in a horse as furosemide. Because furosemide is the drug of choice for treatment of epistaxis or EIPH in the horse, these drugs are sometimes substituted for furosemide in racing horses, especially in jurisdictions where the use of furosemide is restricted. Because both of these drugs are difficult to detect in post-race urine samples and because bumetanide is at least 10 times more potent than furosemide, a need exists for rapid and sensitive tests for these agents. To this end, we raised antibodies to these drugs and developed ELISAs for these diuretics for use in equine medication control.

Both tests are sensitive to the parent drug in the 2-ng/mL range and show virtually no cross-reactivity with pharmacologically or structurally related acidic drugs that may be present in equine urine samples. Based on these useful characteristics of our anti-ethacrynic and anti-bumetanide antibodies, we evaluated their ability to detect the parent drug or drug metabolites in equine serum or urine after administration of these agents to horses.

After administration of 5 mg per horse (approximately 0.012 mg/kg), greater than 80% inhibition of the ELISA was seen for the first 4 hours after dosing, after which the inhibition declined and the ELISA values returned toward control values. Based on these data it should be possible to easily detect this drug in post-race urine samples for up to 4 hours at doses as low as 5 mg ethacrynic acid per horse.

To evaluate the probability of detecting ethacrynic acid administration in routine post-race urine testing, we measured the background inhibition of this ELISA by 40 post-race urine samples from horses racing in Kentucky. As shown in Figure 4, the apparent ethacrynic acid concentrations of these samples (diluted 1:4) were nearly all equal to or

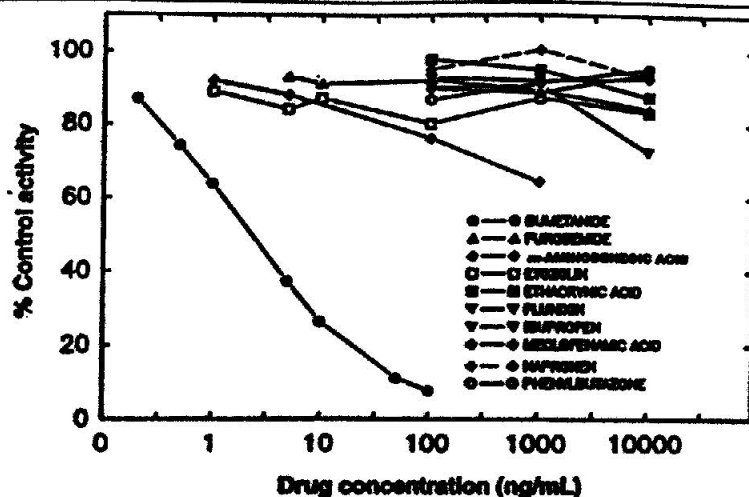


Figure 6. Sensitivity and cross-reactivity of the bumetanide ELISA. Half-maximal inhibition (I-50) occurs at around 2 ng/mL for bumetanide, with no significant cross-reactivity associated with the other drugs tested.

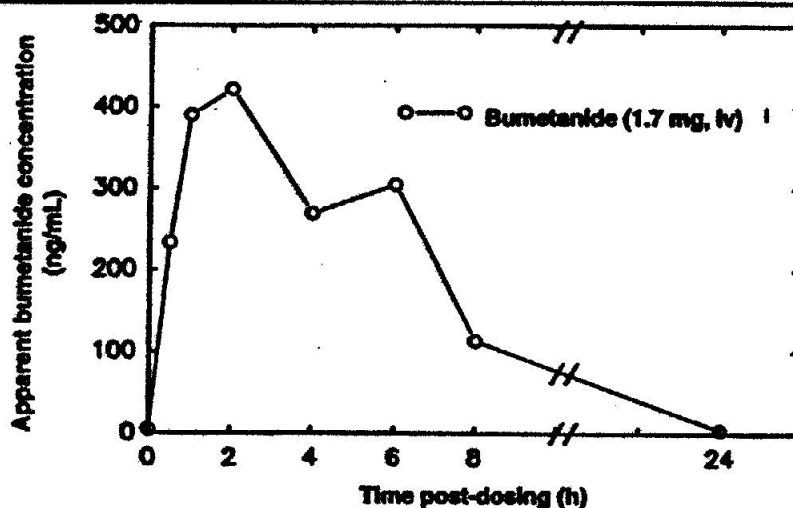


Figure 6. ELISA detection of bumetanide in equine urine. The open circles (O-O) show the calculated apparent bumetanide concentration in urine samples collected after intravenous administration of 1.7 mg bumetanide per horse.

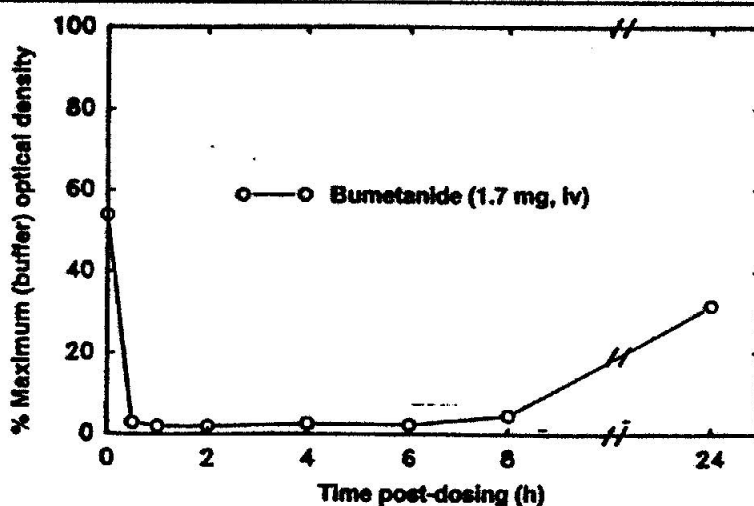


Figure 7. ELISA inhibition by bumetanide dosed-horse urine samples. The open circles (O-O) show the decrease in OD_{450} in urine samples collected after intravenous administration of 1.7 mg bumetanide per horse.

less than the I-50 of the ELISA (2.5 ng/mL). The apparent ethacrynic acid concentration of the diluted dosed-horse urine, indicated by the arrow, resulted in an inhibition of about 80%, which is visually clear (no color). Any lower concentration of ethacrynic acid resulted in the appearance of some color. Therefore, all the diluted track urine "negative" samples were easily distinguished from the "positive" (dosed) drug sample. This ELISA should therefore readily serve as a useful test for ethacrynic acid in post-race urine samples.

The ELISA for bumetanide was also very sensitive to this drug, being half-maximally inhibited by about 2 ng/mL of the parent drug. As with the ethacrynic acid ELISA, little cross-reactivity was seen with other acidic drugs likely to be found in equine samples, suggesting that the test will readily detect

bumetanide in equine blood or urine samples.

The principle problem with the detection of bumetanide is the potency of this drug because its therapeutic dose in a racing horse is approximately one-twentieth of the equivalent dose of furosemide. Because of this, a dose of bumetanide of about 1.7 mg per horse may produce a pharmacological effect, and this small dose may create detection problems for this drug in urine samples.

In equine urine, the period after bumetanide dosing for which 90% inhibition of the ELISA is seen is approximately 2 hours after administration of 1.7 mg per horse. Thereafter, inhibition of the ELISA gradually decays to reach about 40% inhibition at 24 hours after drug administration. These preliminary data therefore suggest that this bumetanide ELISA should readily detect, in post-race urine samples, the administration of doses of bumetanide as low as 1.7 mg per horse for up to 8 hours after administration of this drug.

As was the case with the ethacrynic acid ELISA, the frequency distribution of the apparent bumetanide concentrations of the diluted track urine samples (Figure 8) indicates the ease of visually distinguishing the "negative" samples from a "positive".

Based on their ease of use and sensitivity, these tests would seem to have wide applications in equine drug testing and also in testing of other species. Diuretics such as furosemide, bumetanide, and ethacrynic acid have been used in human athletics in attempts to dilute the urine and reduce the probability of detection of certain drugs; they have also been used in show cattle and other animals to improve the appearance of these animals.

In keeping with these suggestions, recent introduction of these tests into post-race testing in two midwestern states has resulted in five "positives" for ethacrynic acid and at least 13 "positives" for bumetanide in racing horses, thereby enabling control of previously undetected patterns of medication abuse.

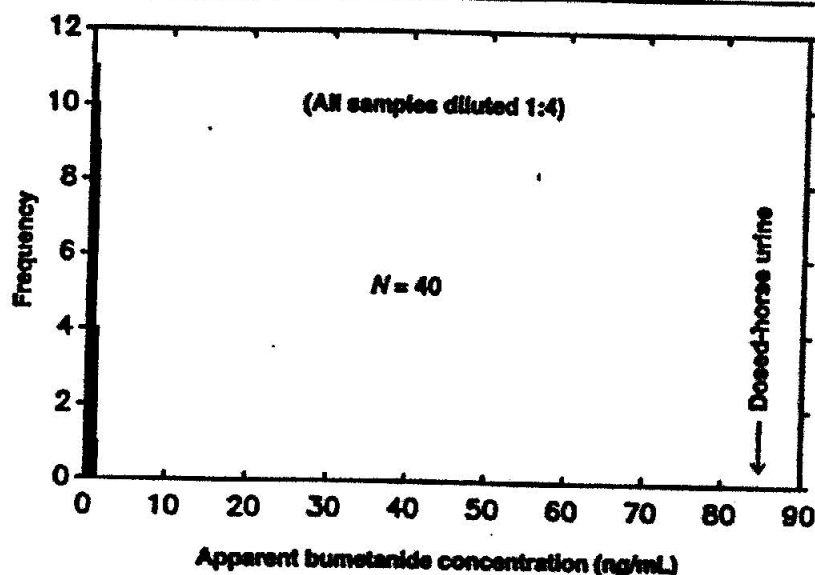


Figure 8. The frequency distribution of apparent bumetanide concentrations for 40 bumetanide negative post-race urine samples. The apparent bumetanide concentration of a sample collected 1 h post-dose from a horse administered 1.7 mg bumetanide intravenously is indicated by the arrow. All samples were diluted 1:4 with assay buffer.

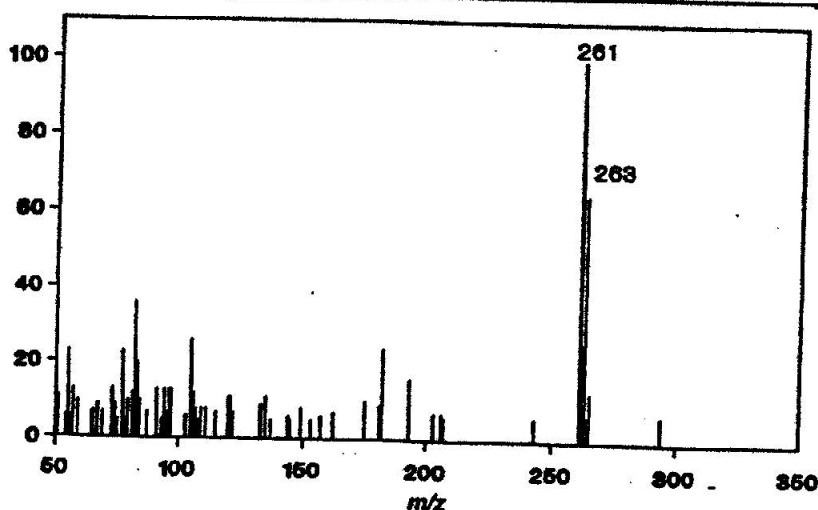


Figure 9. GC/MS profile of an ethacrynic acid urine extract developed as part of a post-race confirmation of an ELISA "positive" for this drug. During the analysis, ethacrynic acid was converted to the methyl ester derivative. The mass-to-charge ratio (m/z) is shown on the x-axis. The y-axis represents the relative abundance of the ions. This MS spectrum was considered absolute identification of ethacrynic acid in the sample.

Acknowledgments

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References

1. D.A. Koechel. Ethacrynic acid and related diuretics. *Ann. Rev. Pharmacol. Toxicol.* 21: 265-83 (1981).
2. A. Ward and R.C. Heel. Bumetanide: A re-

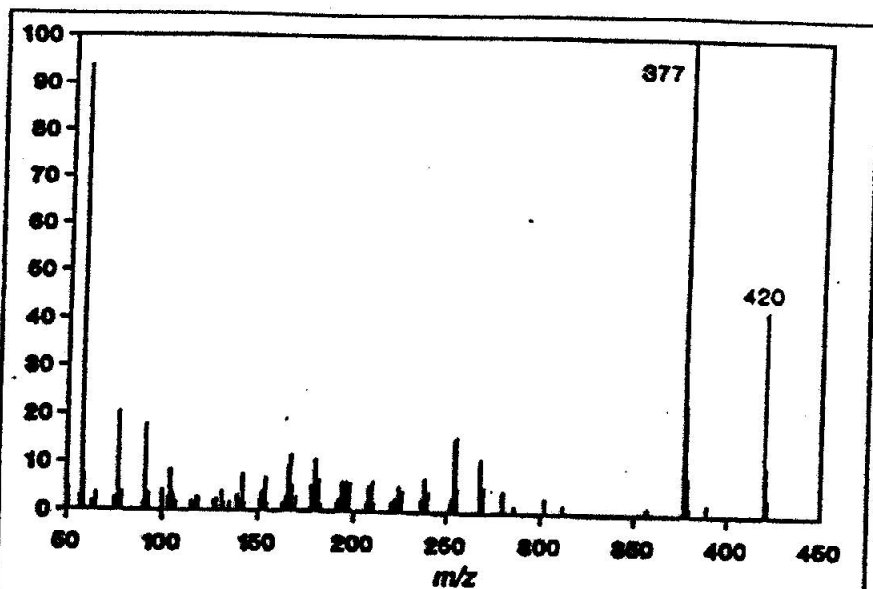


Figure 18. GC/MS profile of a bumetanide urine extract developed as part of a post-race confirmation of an ELISA "positive" for this drug. During the analysis, bumetanide was converted to the tetramethyl derivative. The mass-to-charge ratio (m/z) is shown on the x-axis. The y-axis represents the relative abundance of the ions. This MS spectrum was considered absolute identification of bumetanide in the sample.

- view of its pharmacodynamic and pharmacokinetic properties and therapeutic uses. *Drugs* 28: 428-64 (1984).
3. D.L. Davies, A.F. Lant, N.R. Millard, A.J. Smith, J.W. Ward, and G.M. Wilson. Renal action, therapeutic use, and pharmacokinetics of the diuretic bumetanide. *Clin. Pharmacol. Therap.* 15: 141-55 (1974).
 4. W.R. Olson, R.L. Young, A. Holazo, M.L. Jack, R.E. Weinfeld, K. Alexander, A. Liebman, and S.A. Kaplan. Bumetanide: Radioimmunoassay and pharmacokinetic profile in humans. *J. Pharmacol. Sci.* 65: 701-704 (1976).
 5. J.A. Rudy, T.A. Cochran, T.J. McNicholas, and J.M. Enright. The detection and confirmation of ethacrynic acid in equine urine and plasma. In *AORC 35th Proceedings*. G.H. Johnston, Ed., New Orleans, LA, 1981, pp. 199-210.
 6. W. Stöber, E. Mutschler, and D. Steinbach. Determination of ethacrynic acid and fentanyl in plasma by gas-liquid chromatography-mass spectrometry. *J. Chromatogr.* 227: 193-98 (1982).
 7. B. Ameer and M.B. Burlingame. Determination of bumetanide in human plasma and urine by high-performance liquid chromatography with fluorescence detection. *Anal. Lett.* 21: 1589-1601 (1988).
 8. D.E. Smith. High performance liquid chromatographic assay for bumetanide in plasma and urine. *J. Pharmacol. Sci.* 71: 520-23 (1982).
 9. T. Tobin. *Drugs and the Performance Horse*, Charles C. Thomas, Springfield, IL, 1981, p. 412.
 10. S. Stanley, A. Jeganathan, T. Wood, P. Henry, S. Turner, W.E. Woods, M. Green, H.-H. Tai, D. Watt, J.W. Blake, and T. Tobin. Morphine and etorphine: XIV. Detection by ELISA in equine urine. *J. Anal. Toxicol.* 15: 305-10 (1991).
 11. T. Tobin, H.-H. Tai, C.L. Tai, P.K. Houtz, M.R. Dai, W.E. Woods, T.J. Weckman, J.-M. Yang, S.-L. Chang, J.W. Blake, J. McDonald, R. Gall, P. Wiedenbach, V.D. Bass, B. DeLeon, F.J. Ozog, M.T. Green, C. Brockus, D. Stibert, S. Wie, and C.A. Prange. Immunoassay detection of drugs in racing horses. IV. Detection of fentanyl and its congeners in equine blood and urine by a one step ELISA assay. *Res. Comm. Chem. Pathol. Pharmacol.* 60: 97-115 (1988).
 12. A. Voller, D.W. Bidwell, and A. Bartlett. The enzyme linked immunoassay (ELISA). *Bull. Wild. Hlth. Org.* 53: 55-68 (1976).
 13. G.L. Rowley, K.E. Rubenstein, J. Huisjen, and E.F. Ulman. Mechanism by which antibodies inhibit hapten-maleate dehydrogenase conjugates. *J. Biol. Chem.* 250: 3759 (1975).

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