The Detection of Furosemide in Equine Blood by Fluorescence and Enzyme-Linked Immunoassays

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Abstract

...e step enzyme-linked immunosorbent assay ...LISA) and a particle concentration fluorescence ...noassay (PCFIA) test for furosemide were ...ed as part of a panel of pre- and post-race tests ...gal medication of racing horses. The ELISA ... very sensitive to furosemide with an I-50 for ...ide of about 20 ng/ml. The test is rapid and ...ead with an inexpensive spectrophotometer, ...by eye. Both the PCFIA and ELISA tests ...tect the presence of furosemide in equine blood for up to five hours after administration of the recommended therapeutic dose of this agent. The principal utility of these test lies in rapid screening of furosemide. Thus these tests can be used pre-race to determine whether or not horsemen have treated their horses with furosemide, and post-race to determine whether or not certain plasma concentrations of furosemide have been exceeded. Pilot trials with these systems in Kentucky and Illinois suggest that these tests are economical and effective, and can substitute for the currently used detention barn system of monitoring furosemide administration.

Introduction

Furosemide (Lasix®) is an anthranilic acid derivative (5-(amino-sulfonyl)-4-chloro-2-[(2-furanylmethyl)-amino]benzoic acid). As a high ceiling diuretic furosemide is currently used for the prophylactic treatment of exercise-induced pulmonary hemorrhage (EIPH) or epistaxis ("bleeding") in the race horse (Tobin, 1981; Chay et al., 1983). The incidence of epistaxis in racing horses has been considered to be low; however, recent surveys using fiberoptic endoscopy have indicated as many as 44% up to 75% of Thoroughbred horses examined after racing had blood in their tracheobronchial airways (Clarke, 1986). Occurrence of epistaxis bleeding during a race can cause the affected horse to slow or stop abruptly, posing a serious threat to horses and jockeys in a tightly packed field. In an effort to control this condition during races, many racing jurisdictions allow the pre-race use of furosemide.

It has been reported that a 200 mg/horse intravenous (IV) dose of furosemide given 1 hr pre-exercise may reduce, but not prevent, epistaxis in most horses with a history of EIPH (Pascoe et al., 1985). A furosemide

dose at a similar (0.5mg/kg IV) level has been found by gas chromatographic methods to result in a plasma concentration of about 100 ng furosemide/ml 1 hr post-dose and about 10 ng/ml 4 hr post-dose (Chay et al., 1983). While this treatment may reduce the incidence of EIPH, the cause of EIPH and the mechanism of therapeutic action of furosemide treatment are as yet unknown. Furosemide has been shown to have little or no effect on systemic circulation (Manohar, 1986) or hemostatic function (Kociba et al., 1984) in horses, but does reduce pulmonary arterial pressure (Goetz and Manohar, 1986). It has also been reported that the administration of furosemide to EIPH horses may return a horse to a previous performance level (Soma *et al.*, 1985).

The principal objection to the pre-race use of furosemide is associated with its diuretic response. This diuresis may cause a dilution of illegal drugs and drug metabolites in post-race urine samples, rendering their detection more difficult. Approval of furosemide without regulatory controls may therefore make the task of the racing chemist more difficult.

One way to counteract furosemide's potential interference with drug screening is to avoid urine collection during the period of diuresis. Previous work from our laboratories has shown that furosemide-induced diuresis has a rapid onset and decline (Tobin et al., 1978). After prophylactic doses of furosemide (0.5 mg/kg) administered IV, the diluting effects of furosemide are essentially complete within 3 hr of dosing (Combie et al., 1981). Therefore, furosemide administration at prophylactic doses within 4 hr of post time would cause no dilution of post-race urine samples. This procedure has also been suggested by the Veterinary Chemist's Advisory Committee to the National Association of State Racing Commissioners (NASRC) (Gabel et al., 1977).

The enforcement of time rules for furosemide is commonly achieved by means of a detention barn system. In this system, horses to be treated with furosemide are stabled in a secure barn about 5 hr before race time. Furosemide is administered at the approved dose and time pre-race under constant supervision. Although highly visible and effective, such systems are expensive and may not be justified by the potential magnitude of the drug-diluting effect observed in practice (Tobin, 1981). A less expensive alternative approach to enforce compliance with a "4 hr furosemide" rule could be to designate a plasma "tolerance level" of furosemide, above which there would be a substantial probability of violation of the time rule. The frequency distribution of furosemide plasma levels at 4 hr after administration of the recommended therapeutic dose would be determined and used as the basis for determining this tolerance level.

A data base on which such a tolerance level could be determined has been developed by Tobin and his co-workers (Chay et al., 1983). This data shows that if doses of 0.5 mg/kg are administered IV to horses there is less than one chance in one thousand of a plasma level of 30 ng/ml being exceeded at 4 hr post-dose. Thus, one regulatory strategy is to monitor plasma levels of furosemide post-race, and discipline those whose post-race plasma levels exceed a stipulated level such as 30 ng/ml.

Such a program is now in place in harness racing in Kentucky, and is functioning very satisfactorily. However, because of the inconvenience and expense of estimating post-race plasma levels of furosemide by high performance liquid chromatography (HPLC), we have developed a simple one step enzyme-linked immunosorbent assay (ELISA) and a particle concentration fluoroimmunoassay (PCFIA) to detect and quantitate furosemide in equine blood.

Material and Methods

Horses

Mature Thoroughbred, half Thoroughbred, and Standardbred mares (400-600 kg) were used throughout. The animals were kept at pasture and allowed free access to food and water. The horses were placed in standard box stalls (17 m^2) approximately 12 hours prior to dosing for acclimatization.

Serum from racing horses was collected under the authority of the officials in charge at the individual racecourses after races and delivered to the drug testing laboratory of the racing jurisdiction.

Dosing and Sampling

Authentic furosemide standard was obtained from

Sigma Chemicals (St. Louis, MO). Furosemide was administered as Lasix® (5% solution, American Hoechst, Somerville, NJ) by rapid injection into the jugular vein (IV).

Blood samples were collected by venipuncture into vacuum blood tubes containing potassium oxalate and sodium fluoride (for plasma) or serum separation vacuum tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ). The blood tubes were centrifuged to collect the plasma and the serum. All samples were aliquoted and stored frozen until assayed.

One Step ELISA

One step ELISA tests were performed as previously described (Yang et al., 1987; McDonald et al., 1988; Tobin et al., 1988; Prange et al., 1987). Briefly, anti-furosemide antibody was linked to flat bottom Immulon Removawells® (Dynatech, Chantilly, VA) (Voller et al., 1976). Rabbit anti-furosemide antibody was produced as previously described (Woods et al., 1986; Tai et al., 1986). Furosemide was linked to horseradish peroxidase (HRP) (Pradelles et al., 1985), to give rise to a covalently bound furosemide-HRP complex.

The assay was started by adding 20 µl of the standard, test, or control samples to each well, along with 100 µl of the furosemide-HRP solution. Furosemide standards were prepared in PBS buffer (phosphate buffered saline, 0.10M sodium phosphate, pH 7.4 Sigma Diagnostics, St. Louis, MO). During this step, the presence of free drug competitively prevented the antibody from binding to the furosemide-HRP conjugate. The degree of the antibody-furosemide-HRP binding was therefore inversely proportional to the amount of drug in the sample. After 15 min of incubation the fluid was removed from the microtiter wells and the wells washed twice with buffer. TMB Microwell Peroxidase substrate solutions (Kirkegaard and Perry, Gaithersburg, MD) were than added to all wells and their optical density read at 650 nm in an Ultrascan microwell reader (International Diagnostic Systems Corp., St. Joseph, MI) at 30 min after addition of substrate.

Particle Concentration Fluorescence Immunoas (PCFIA)

PCFIA studies were performed as previously descril (Jolley et al., 1984; McDonald et al., 1987; Yang al., 1987) on a Pandex Fluorescence Concentrati Analyzer (Pandex, Mundelein, IL). The ba functional unit in the Pandex PCFIA is a 96 well pl with a filter base in each plate. To each well is add 20 µl of furosemide-B-phycoerythrin (furosemi BPE), 40 µl of anti-furosemide antibody, and 40 of blank, standard, or test sample. The system is allow to equilibrate for about 10 minutes after which a seco antibody system is added. The second antibody consi of goat anti-rabbit antibody bound to latex beads. T system is allowed to react for another 10 minutes a then the fluid is drawn out of the system through t filter membrane. The reaction system is washed wi about 80 µl of phosphate buffer to resuspend t particles, and the system again drawn down with t vacuum. The filtration step has the effect of conce trating the latex beads 1000-fold, thereby increasi the sensitivity of the method. After the wash step, t fluorescence of the particles at 545 and 575 nm measured. The mean response from control urines usually about 25,000-30,000 arbitrary fluorescen units/well.

Mass Spectral Confirmation of Furosemide

The presence of furosemide in serum samples detecte by PCFIA was confirmed by gas chromatography mass spectroscopy (GC/MS) of a methylated furosem ide derivative. The serum sample (5-10 ml) was mad acidic with 4 ml of potassium phosphate (KH_2PO_4 H_3PO_4) buffer, pH 3.0, and extracted with 6 m dichloromethane (DCM). The sample was mixed b rotation for 5 min. The DCM phase was isolated b centrifugation, and evaporated to dryness under N in a 35° C water bath.

The residue after evaporation was dissolved in 50 µ methyl acetate and "streaked" on a 0.25 mm silica ge thin layer chromatography plate, along with furosemide standard and a negative control. The thre "streaks" were eluted from the adsorbent with methanol which was reduced to dryness under N_2 at 35° C. Th residue was dissolved in 20 µl methanol and 10 µ trimethylanilinium hydroxide (MethElute, Pierce Rockford, IL).

The methylation took place "on column" as the sample was injected onto a capillary GC (Model #5890, Hewlett-Packard Instruments, Palo Alto, CA) equipped with MS detector and data station (Hewlett-Packard). GC/MS conditions were similar to those previously described (McDonald et al., 1988).

Results

The data of Figure 1 show the inhibition of the ELISA reaction by added furosemide. Increasing concentrations of furosemide inhibited the reaction, with maximal inhibition occurring after addition of about 200 ng/ml of furosemide to the system. Figure 2 shows the same data plotted as dose response curves. Half maximal inhibition was obtained at about 20 ng/ml added to the system, so this ELISA reaction is well configured to determine the presence or absence of concentrations of furosemide in the order of about 30 ng/ml. Since all of the reading curves obtained at different time points overlap, Figure 2 shows that the time at which the reaction is read does not appear to affect the apparent sensitivity of the reaction.

Figure 3 shows the ability of this test to detect furosemide in equine plasma after administration of the recommended doses of furosemide to five horses. Immediately after administration of furosemide the ELISA test was essentially completely inhibited, indicating levels of furosemide in these samples exceeding 200 ng/ml. By about one hour after dosing, plasma levels of furosemide had dropped to about 100 ng/ml, and the inhibition of the ELISA test is no longer complete. Thereafter, as the levels of furosemide in the plasma of these horses falls, the ELISA becomes less inhibited, and at 4 hr after dosing is about 65% inhibited. This represents the degree of inhibition that one would expect to find in the "average horse", and a plasma sample containing 30 or 50 ng/ml of furosemide can be added to the test system to enable rapid estimation of whether or not a regulatory level has been exceeded.

A broadly similar pattern of inhibition of this ELISA test is also readily apparent in serum samples from these same horses (Figure 4). Based on these data, either plasma or serum could be used as a regulatory tool to perform preliminary screening to determine whether or not a regulatory level of furosemide has been exceeded.

A PCFIA standard curve developed for furosemide (Figure 5) was determined to be log linear with half maximal inhibition of the fluorescence reaction occurring about 20 ng/ml of furosemide. In comparison to the ELISA standard curve (Figure 1), the PCFIA appears to be of about equal sensitivity. Based on the sensitivity of the PCFIA it should be possible to use this test to determine the presence or absence of furosemide in a sample relatively rapidly.

The PCFIA test was evaluated as part of a horse racing pre-race testing program for furosemide (Table 1). Two "known" (provided by the analyst) and 7 "blind" (unknown to the analyst) quality assurance furosemide serum samples were mixed in with pre-race serum samples provided by race track officials. These samples were screened for their furosemide status by PCFIA, those flagged "positive" were analyzed by GC/MS. Out of 9 quality assurance tests, the PCFIA flagged 8 samples of which 7 were confirmed by GC/MS to contain furosemide. The complete PCFIA-GC/MS processes were completed within 1 hr, 40 min, demonstrating the ability of this technology to screen for the presence of a drug and confirm the identification by GC/MS within the 120 min limit set by a pre-race testing program.

Discussion

The ELISA test reported here offers a rapid, simple, and inexpensive method of screening for furosemide in equine blood samples. The method is comparable in its characteristics to the other ELISA tests that have been reported (Yang et al., 1987; Tobin et al., 1988; Prange et al., 1988; McDonald et al., 1988). Its sensitivity, development time, and ease of use are all exactly the same as those of previously reported tests. However, the principal difference between this ELISA test for furosemide and the other tests is that furosemide is a legal drug in most racing jurisdictions in North America. The major utility of this test, therefore, depends on whether it can be used to determine if furosemide has in fact been used in compliance with the rules that govern its use.

The PCFIA test is also a rapid and efficient method for the quantitation of furosemide in equine blood samples. This assay technique was successfully evaluated in a pre-race quality assurance testing

program for furosemide. The objective of this test was to analytically confirm compliance with the regulations governing the use of furosemide. In Illinois, where the PCFIA-furosemide tests were carried out, furosemide is required to be administered 4 hr prior to post time at a dosage rate of 250 mg/horse intravenously.

The potential for application of a simple quantitative test for furosemide is large. The regulations which govern the use of furosemide may be grouped into three broad categories. The first method is the honor system. Under this rule horsemen are simply informed that the legal dose of furosemide is 250 mg/horse at not less than fours before post time. No further efforts at monitoring compliance with the rule are made.

The second method is the detention barn system. Under this system the horses are brought to the detention barn and the drug may be administered by the Racing Commission Veterinarian. Thereafter the horse is maintained in the detention barn or in a specified stall barn under some degree of supervision. The objective is to ensure that a second dose of furosemide is not given to the horse, which might be used to dilute out or mask a potent illegal medication in the horse's post-race urine samples. While much more rigorous than the honor system, there is no information whatsoever as to the effectiveness of this detention barn system. Its expense, however, can readily be calculated in terms of the actual costs of real estate and security guards. Beyond this another cost exists, which is the cost to the horsemen of moving their horses just prior to a race and subjecting them to an upsetting stress. For these reasons the detention barn is a less than ideal solution to the problem of the diuretic effects of furosemide.

The data reported here from the Illinois pre-race testing program shows that the detention barn is a less than ideal solution to the problem. For whatever reason, the detention barn system enables a small percentage of horsemen to avoid running their horses on furosemide when they are in fact declared as being on furosemide. Similarly, it appears that horses not declared as being on furosemide are in fact running on furosemide. These data strongly suggest that the best way of ensuring compliance with the furosemide rule is by determining the chemical status of the animal, rather than by attempting to maintain a constant watch over a relatively large number of horses for several hours at a time.

Our experience in Kentucky with the pilot program using a quantitative level for furosemide in horses has been very successful. In the first place this program allows one to unequivocally determine the status of a horse with respect to furosemide. A detention barn system merely indicates the previous history of the horse, and is only suggestive of what its actual furosemide status may be. As shown in Illinois analytical monitoring is the only ultimately satisfactory method of determining the furosemide status of a horse.

The principal problem with analytical monitoring for furosemide has been the instrumental and labor cost involved. The first useful quantitative analytical method for furosemide, reported by Tobin and co-workers (Chay et al., 1983) required a level of analytical skill which was apparently not readily available, even in laboratories specializing in equine forensic chemistry. Since then more easily reproduced methods based on HPLC analysis have become available, but these methods still require expensive instrumentation and skilled operatives. A simple quantitative screening method for furosemide is therefore desirable, and would render quantitative analysis for furosemide a much more economical and practical proposition.

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Figure 1. *The symbols show the time course of color development in the presence of increasing concentrations of furosemide in PBS buffer.*

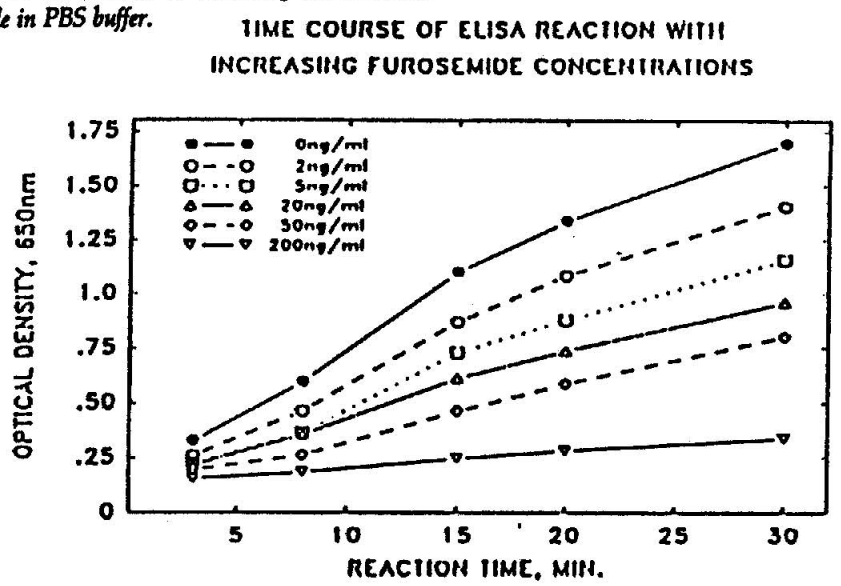


Figure 2. *The symbols show dose response curve representations of the data of Figure 1 indicating that half maximal inhibition of the reaction occurs at about 20 ng/ml. Maximum optical density was the reading for PBS buffer with no drug added.*

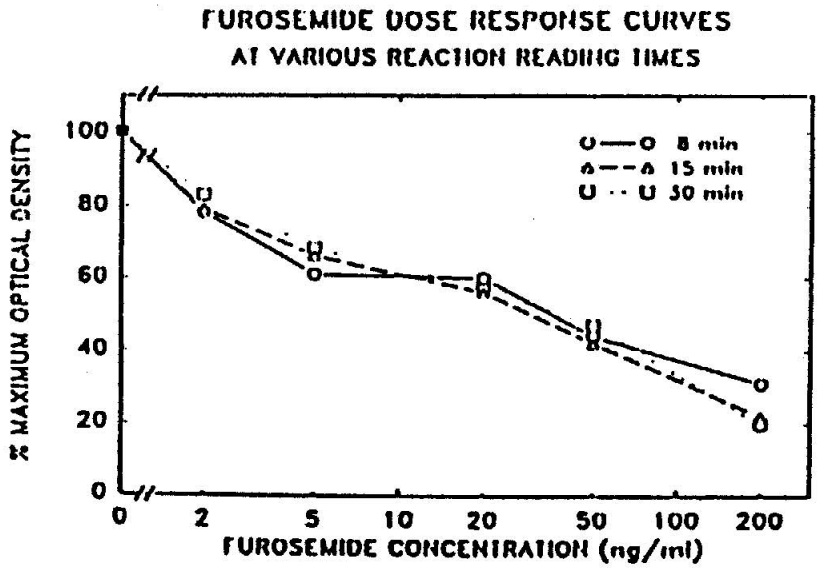
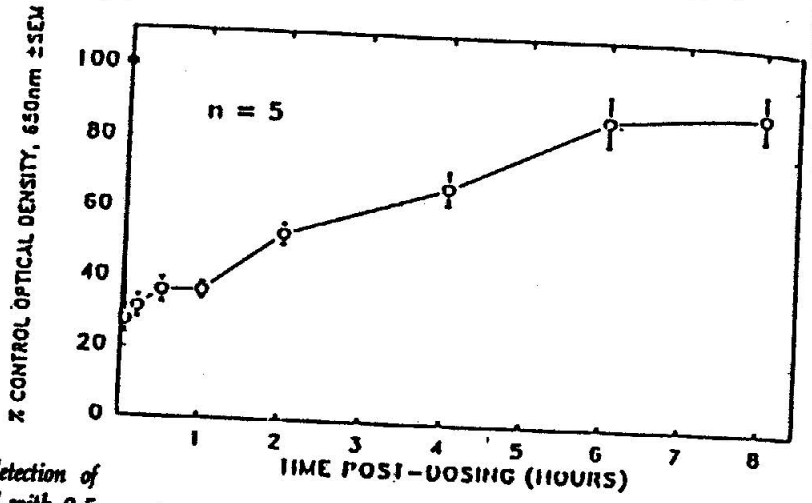


Figure 3. *The figure shows the ELISA detection of furosemide in the plasma from five horses dosed with 0.5 mg/kg of furosemide IV. Reaction reading time = 30 min. Control optical density was the mean reading for the pre-dose samples.*



gure 4. *The figure shows the ELISA detection of osemide in the serum from five horses dosed with 0.5 /kg of furosemide IV. Reaction reading time = 30 n. Control optical density was the mean reading for pre-dose samples.*

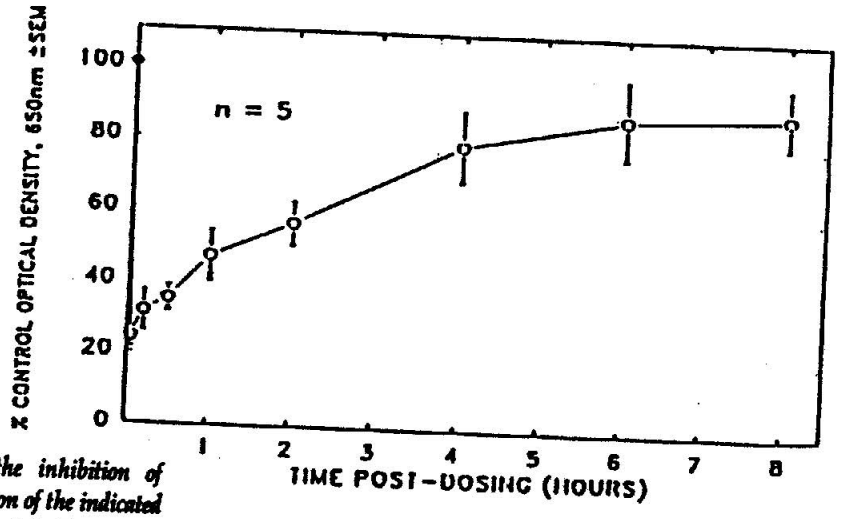


Figure 5. *The standard curve for the inhibition of furosemide-BPE fluorescence by the addition of the indicated concentrations of furosemide was constructed. Furosemide (1-100 ng/ml) was added to normal race track serums which were diluted 1:10 for assay. No extractions were conducted.*

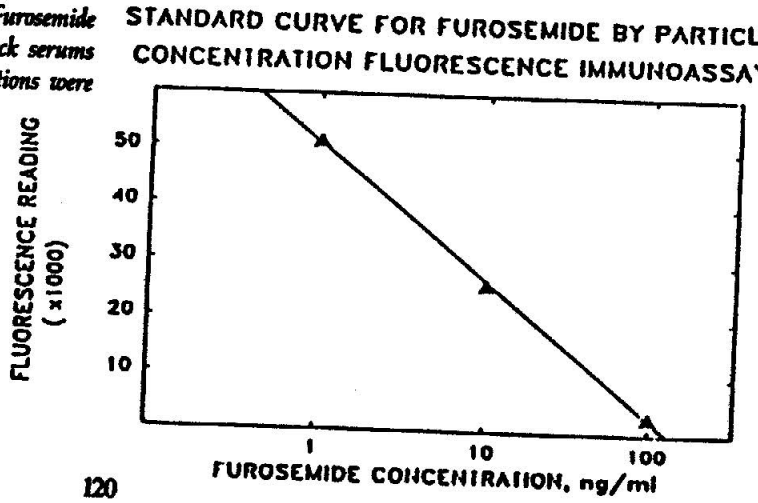


Table 1. *Pre-race test*

Test #	Sampling
1	1.25 hr
2	1.26 hr
3	1.22 hr
4	1.25 hr
5	2.00 hr
6	2.00 hr
7	0.50 hr
8	0.58 hr
9	1.30 hr

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Table 1. *Pre-race testing quality assurance samples.*

Test #	Sampling Time	Type	Tests	Results	Analysis Time
1	1.25 hr	Known	PCFIA GC/MS	Positive Positive	1 hr, 33 min
2	1.26 hr	Known	PCFIA GC/MS	Positive Positive	1 hr, 31 min
3	1.22 hr	Blind	PCFIA	Negative*	
4	1.25 hr	Blind	PCFIA GC/MS	Positive Positive	1 hr, 34 min
5	2.00 hr	Blind	PCFIA GC/MS	Positive Inconclusive**	1 hr, 33 min
6	2.00 hr	Blind	PCFIA GC/MS	Positive Positive	1 hr, 38 min
7	0.50 hr	Blind	PCFIA GC/MS	Positive Positive	1 hr, 26 min
8	0.58 hr	Blind	PCFIA	Positive 1 hr, 25 min	
9	1.30 hr	Blind	GC/MS PCFIA GC/MS	Positive Positive	1 hr, 24 min

Quality assurance serum samples were those collected at various sampling times post-dose from horses intravenously dosed with 250 mg furosemide. The quality assurance samples, either added by the analyst (known) or submitted to the analyst along with the track samples (blind), were analyzed for furosemide with pre-race track serum samples. Those samples flagged "positive" by PCFIA were assayed by GC/MS for confirmation. Length of time of the total analysis is shown for each example.

*PCFIA was repeated with a positive result the second time. The GC/MS was determined to be inconclusive.

**GC/MS was inconclusive after 2 runs. PCFIA was positive on a second test.

Discussion

CHARLES PRANGE Could you review the McDonald data again? On the Lasix study, were those spiked serums?

TOM WOOD I believe they were spiked.

CP How come the one was negative on both immunoassay and GC/MS? Are you sure those were spiked and not actual administrations on horses running race in Chicago?

TW I believe those were spiked samples but now I could be wrong, you probably would have to talk to Dr. McDonald.

CP I'm almost positive those were live samples from the lasix detention barn at Sportsman's Park

ROBERT McKENZIE You claim economy in these tests, could you give us a feel for what economy is?

TW Compared to the man hours of the other tests that are involved, the initial cost is going to be reasonably expensive. I don't have the figures of the cost of the test myself but as far as man hours and the time involved to do the test, that is what I mainly referred as far as economical.

GERRY JOHNSTON I notice that you mentioned the 30 nanogram level, is the actually a rule being applied in Kentucky racing?

TW Yes, in the harness racing it is the rule. In Thoroughbreds it is a little more liberal; it is more the honor system.

GJ Are you equating that 30 nanograms to a four hour administration?

TW Yes, this test would easily pick up a concentration that high or even higher.

RANDY LEAVITT I wonder if you could tell me, Dr. Wood, what substrate you are using for HRP reaction.

TW It is just a peroxidase substrate, I'm not sure of the exact . . . you mean the fluorescence assay?

RL No, for the horseradish peroxidase.

TW It is just a peroxidase substrate; I'm not exactly sure of the substrate.

RL Could you direct me as to who might know? The man right behind you I believe has the answer.

SIONG WIE It is PMB.