IMMUNOASSAY DETECTION OF DRUGS IN RACING HORSES
VI. DETECTION OF FUROSEMIDE (LASIX[R]) IN EQUINE BLOOD
BY A ONE STEP ELISA AND PCFIA

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SUMMARY

A one step enzyme-linked immunosorbent assay (ELISA) and a particle concentration fluorescent immunoassay (PCFIA) test for furosemide were evaluated as part of a panel of pre- and post-race tests for illegal medication of racing horses. These tests are very sensitive to furosemide with an I-50 for furosemide of about 20 ng/ml. The test is also rapid; an average pre-race complement of 10 samples can be analyzed in 90 minutes or less. The ELISA test results can be read with an inexpensive spectrophotometer, or even by eye. Both the PCFIA test and the ELISA test readily detect 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 tests lies in rapid screening of samples for compliance with regulations governing the use of furosemide. Thus these tests can be used pre-race to determine whether horsemen have treated their horses with furosemide, and post-race to perform an intial evaluation of whether certain blood 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 form part of an analytical approach to substitute for the detention barn system of monitoring furosemide administration.

INTRODUCTION

Furosemide (Lasix[R]) 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

racehorse (Tobin, 1981; Chay et al., 1983). The incidence of epistaxis in racehorses has been considered to be low; however, recent surveys using fiberoptic endoscopy have indicated as many as 44% to 75% of Thoroughbred horses examined after racing had blood in their tracheobronchial airways (Clarke, 1986). Occurrence of epistaxis during a race can cause the affected horse to slow or stop abruptly, posing a serious threat to horse 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 (0.5 mg/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 administration of furosemide to EIPH horses may return them to previous performance levels (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 IV), diluting effects are essentially complete within 3 hr of administration (Combie et al., 1981). Therefore, furosemide adminstration 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 alternate 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). The data show 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.

MATERIALS AND METHODS

Horses

Mature Thoroughbred, half Thoroughbred and Standbred 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 as 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[R] (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.

<u>One Step ELISA</u>

One step ELISA tests were performed as previously described (Yang <u>et al</u>., 1987; McDonald <u>et al</u>., 1988; Tobin <u>et al</u>., 1988; Prange <u>et al</u>., 1988). Briefly, anti-furosemide antibody was linked to flat bottom Immulon Removawells[R] (Dynatech, Chantilly, VA) (Voller <u>et al</u>., 1976). Rabbit anti-furosemide antibody was produced as previously described (Woods <u>et al</u>., 1986; Tai <u>et al</u>., 1986). Furosemide was linked to horseradish peroxidase (HRP) (Pradelles <u>et al</u>., 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 then 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.

Kits for this furosemide test and for other tests reported in this series of papers are commercially available from International Diagnostic Systems Corporation, St. Joseph, Michigan.

Particle Concentration Fluorescence Immunoassay (PCFIA)

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

Mass Spectral Confirmation of Furosemide

The presence of furosemide in serum samples detected by PCFIA was confirmed by gas chromatography/mass spectroscopy (GC/MS) of a methylated furosemide derivative. The serum sample (5-10 ml) was made acidic with 4 ml of potassium phosphate (KH_2PO_4-H_3PO_4) buffer, pH 3.0 and extracted with 6 ml dichloromethane (DCM). The sample was mixed by rotation for 5 min. The DCM phase was isolated by centrifugation, and evaporated to dryness under N_2 in a 35°C water bath.

The residue after evaporation was dissolved in 50 µl methyl acetate and "streaked" on a 0.25 mm silica gel thin layer chromatography plate, along with a furosemide standard and a negative control. The three "streaks" were eluted from the adsorbent with methanol, which was reduced to dryness under N_2 at 35°C. The residue was dissolved in 20 µl methanol and 10 µl trimethylanilinium hydroxide (MethElute, Pierce, Rickford, 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 a 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 on 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
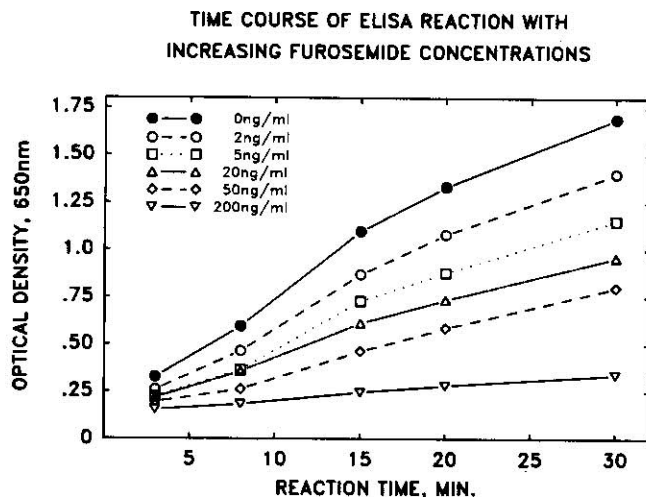
TIME COURSE OF ELISA REACTION WITH
INCREASING FUROSEMIDE CONCENTRATIONS

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

FUROSEMIDE DOSE RESPONSE CURVES
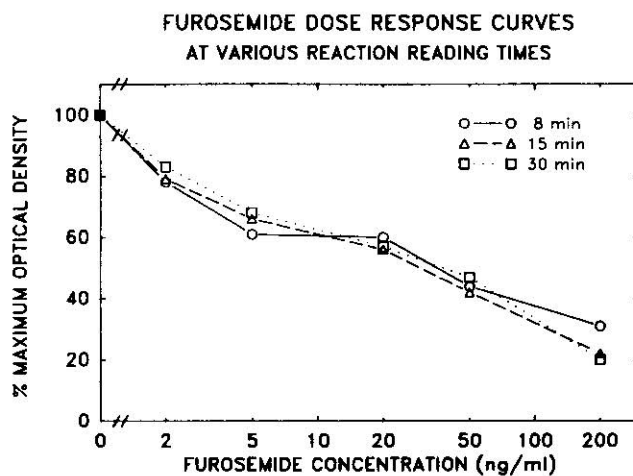AT VARIOUS REACTION READING TIMES

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.

DETECTION OF FUROSEMIDE IN EQUINE PLASMA
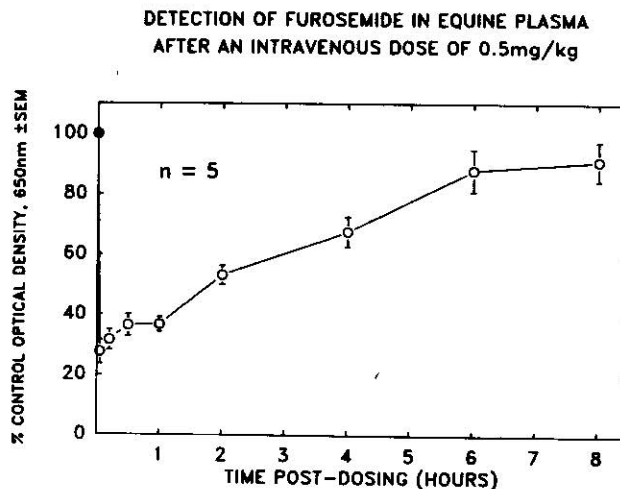AFTER AN INTRAVENOUS DOSE OF 0.5mg/kg

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.

ELISA test is no longer complete. Thereafter, as the levels of furosemide in the plasma of these horses falls, the ELISA test 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 5 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.

DETECTION OF FUROSEMIDE IN EQUINE SERUM
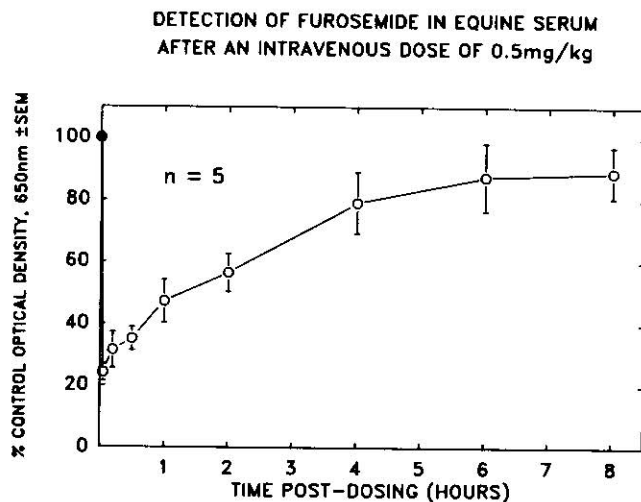AFTER AN INTRAVENOUS DOSE OF 0.5mg/kg

Figure 4. The figure shows the ELISA detection of furosemide in the serum 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.

A PCFIA standard curve developed for furosemide (Figure 5) was determined to be log linear with half maximal inhibition of the fluorescence reaction occurring at about 20 ng/ml of furosemide. In comparison to the ELISA standard curve (Figure 2), 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.

STANDARD CURVE FOR FUROSEMIDE BY PARTICLE
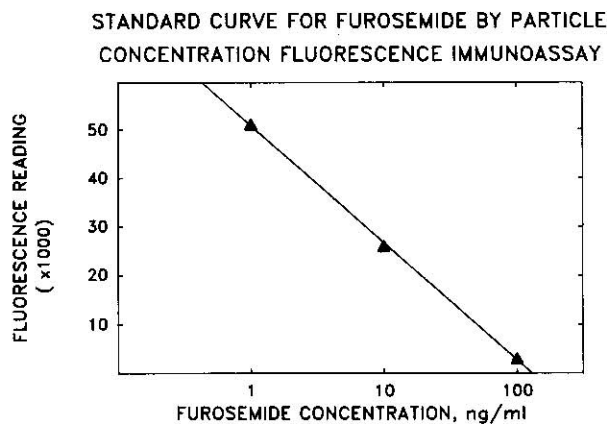CONCENTRATION FLUORESCENCE IMMUNOASSAY

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 mg/ml) was added
to normal race track serums which were diluted 1:10 for assay.
No extractions were conducted.

The PCFIA test was evaluated as part of an equine 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 included 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.

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

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

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 we have 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 the same as those of our previously reported tests. However, the principal difference between this ELISA test for furosemide and the other tests that we have reported is that furosemide is a legal drug in most

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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 given in compliance with the rules that govern its administration.

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 IV.

The application potential 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 administered not less than four hours 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 costs of real estate and security

personnel. Beyond this, another cost exists, which is the cost to the horsemen of moving their horses to unfamiliar surroundings 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 are a further indication that the detention barn is a less than ideal solution to the problem. For whatever reason it has been found that the detention barn system enables a small percentage of horsemen to avoid racing their horses on furosemide when they are in fact declared as being on furosemide. Conversely, it appears that horses not declared as being on furosemide are in fact racing 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.

In Kentucky experience with a pilot program using a post-race quantitative blood level for furosemide in horses has been very successful. 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 by experience in Illinois, analytical monitoring is ultimately the only satisfactory method of determining the furosemide status of a horse.

Heretofore the principal problem with analytical monitoring for furosemide has been the instrumental and labor costs involved. For example the first useful quantitative analytical method for furosemide, reported by Tobin and co-workers (Chay et al., 1983) required a level of analytical

skill not readily available, even in laboratories specializing in equine forensic chemistry. Since then more easily reproduced methods based on high performance liquid chromatographic analysis have become available. These methods still require expensive instrumentation are time comsuming, requiring skilled, and therefore expensive, technicians. A simple, rapid, quantitative screening method for furosemide as reported here is therefore desirable, and would render routine quantitative analysis for furosemide a more economical and practical proposition.

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